PATHOGENESIS OF FROG VIRUS 3 (*Ranavirus* sp, Iridoviridae) IN THE WOOD FROG, *Rana sylvatica* (*Lithobates sylvaticus*)

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of Doctor of Philosophy

Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

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Charlottetown, P. E. I.

October 2015

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ABSTRACT

Amphibian populations suffer massive mortalities from infection with Frog Virus 3 (FV3, Ranavirus, Iridoviridae), a pathogen also responsible for mortalities of fish and reptiles. Wood frogs, *Rana sylvatica*, have been proposed as a research model to study diseases of native amphibians in North America. Our objectives were: 1) describe the long-term housing, feeding and veterinary care of wild-caught wood frogs, 2) develop a Ranavirus-wood frog model of infection with FV3, 3) review the literature on amphibian clinical pathology, 4) determine hematological reference intervals (RIs) for adult wood frogs and the hematological alterations associated with infection with FV3, and 5) establish the chronology of lesions due to a lethal infection with FV3.

Wild-caught adults and tadpoles were collected from Prince Edward Island and maintained in captivity for up to a year. Survival was good for wild-caught individuals: 75 % for wild-caught adults and 77 % for tadpoles raised to adulthood.

A dose trial on adults raised from wild-caught tadpoles established a lethal dose 50 (LD_{50}) of $10^{2.93\ (2.42\text{-}3.44)}$ pfu of FV3 for frogs averaging 35 mm in length. Onset of clinical signs occurred 6-14 days post-infection (dpi) (median 11 dpi) and time-to-death 10-14 dpi (median 12 dpi). Each ten-fold increase in virus dose increased the odds of dying by 23-fold and accelerated onset of clinical signs and death by approximately 15%. Ranavirus DNA was demonstrated in skin and liver of all frogs that died or were euthanized because of severe clinical signs. Shedding of virus occurred in feces (7-10 dpi; 3-4.5 d before death) and skin sheds (10 dpi; 0-1.5 d before death) of some frogs that died from infection. Most common lesions were dermal erosion and hemorrhages, hematopoietic necrosis in bone marrow, kidney, spleen and liver, necrosis in renal glomeruli and in tongue, gastrointestinal tract, and urinary bladder mucosa. Intracytoplasmic inclusion bodies (probably viral) were present in the bone marrow and the epithelia of the oral cavity, gastrointestinal tract, renal tubules and urinary bladder.

A time-course trial on wild-caught adults using a lethal dose of FV3 ($10^{4.43}$ pfu/frog) followed by euthanasia at 0.25, 0.5, 1, 2, 4, 9 and 14 dpi established pathogenesis and

hematological alterations due to infection. Infection with FV3 caused neutrophilia, increase in undifferentiated blast-like cells and relative reduction of basophils. Lymphocytes decreased at 4 and 9 dpi but increased 14 dpi. From 9 dpi onwards, nuclear deterioration and mild toxic change were present in neutrophils; cytoplasmic inclusion bodies were present in lymphocytes, monocytes, neutrophils and eosinophils. FV3 first targets hematopoietic tissue in the bone marrow and endothelial cells in the skin causing very mild microscopic lesions (1-2 dpi). Approximately 9 dpi, FV3 caused severe lesions in medullary and extamedullary hematopoietic tissue, lymphoid tissue and epithelial cells of skin and mucosae throughout the body. Direct contact (skin) and fecal-oral contamination are likely effective routes of transmission.

ACKNOWLEDGMENTS

Funding for this project was provided by the Canadian Wildlife Health Cooperative (formerly Canadian Cooperative Wildlife Health Centre), and by the National Sciences and Engineering Research Council of Canada as an Alexander Graham Bell Canada Graduate Scholarship D and a Michael Smith Foreign Study Supplement.

Numerous people provided professional, technical and in-kind support that was crucial to the successful completion of this project. Their names and the nature of their contributions are listed at the end of each chapter. Others, whose support was not directly associated with the scientific work but which was even more significant, can only be acknowledged here. My mom, who loved me and would have liked to see me finish. My dad, who trusts and, most of the time, understands me. Old friends, who are here for the long run. And Raphaël, for whom there are no words.

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ABBREVIATIONS

IHC (immunohistochemical)

PCV (packed cell volume)

PCR (polymerase chain reaction)

PFU (plaque-forming units)

RBC (red blood cell)

RI (reference interval)

WBC (white blood cell)

INTRODUCTION

> ***Amphibians:*** *This class of animals is distinguished by a body cold and generally naked; a countenance stern and expressive; voice harsh; colour mostly lurid, and filthy odour: a few are furnished with a horrid poison […]; some undergo a metamorphosis […]; some appear to live promiscuously on land or in the water, and some are torpid during the winter.*
>
> Carolus Linnaeus (1758) as translated by William Turton (1800)

Although when Linnaeus wrote his definition of the Amphibias he was also thinking of reptiles, which he placed in the same class (Linnaeus, 1800), his description conveys a slight distaste for cold and slimy creatures. Fortunately, from the early days of modern Biology other naturalists have found amphibians both amenable experimental subjects and an intriguing group of vertebrates in their own right. Studies into the taxonomy and, later on, anatomy and physiology of amphibians flourished in the 18th and 19th centuries. In the 20th century studies on amphibian physiology, embryology, immunology and toxicology multiplied along with an increased interest in the natural history and ecology of wild populations (Duellman & Trueb, 1994). In the 1970s, naturalists and field researchers working in the temperate forests of Australia and Central America began noticing severe decreases in some of the amphibian populations they had studied for years. For two decades, while multiple theories were proposed but none substantiated, the declines continued and some turned into extirpations or extinctions. Finally, in an example of effective trans-disciplinary collaboration and through the rigorous diagnostic investigation of mortality events, the infectious disease chytridiomycosis was found to be responsible for the declines (Berger *et al.*, 1998; Wake & Vredenburg, 2008). Since then *Batrachochytrium dendrobatidis*, the fungus that causes chytridiomycosis, has been recognized as an emerging pathogen worldwide and its impact on frogs as “the most spectacular loss of vertebrate biodiversity due to disease in recorded history” (Skerratt *et al.*, 2007).

The discovery that an infectious agent had played a significant part in the amphibian population crisis of the late 20th century propelled a growth in research on amphibian diseases. It also prompted the involvement of diagnosticians, microbiologists and veterinarians in amphibian research, a field that had been the almost exclusive province of herpetologists and experimental physiologists. The involvement of veterinary researchers in the investigation of frog and salamander mortalities in Australia, Europe and North America resulted in the identification of another emerging pathogen: the *Ranavirus* genus of the Iridoviridae family (Cunningham *et al.*, 1996; Bollinger *et al.*, 1999; Jancovich *et al.*, 1997; Speare & Smith, 1992).

The Iridoviridae is a family of large double-stranded DNA viruses that infect insects (*Iridovirus* and *Chloriridovirus* genera) and poikilothermic vertebrates (*Lymphocystivirus* and *Ranavirus* genera) (Chinchar *et al.*, 2002). The type species of the *Ranavirus* genus, the rather modestly named "Frog Virus 3" (FV3), was first isolated and described by Dr Allan Granoff while investigating the role of viruses in the development of renal carcinomas of Northern leopard frogs, *Rana pipiens*, in the 1960s (Granoff *et al.*, 1966). At the time of its first isolation FV3 was found to have no association with neoplasia formation or any known clinical disease, and so it was considered an "orphan virus" (Chinchar, 2002). In the last two decades, as FV3 and other ranaviruses have caused mortalities in wild and captive amphibians, fish and reptiles (Chinchar & Waltzek, 2014), they have been recognized as "emerging cold-blooded killers" (Chinchar, 2002). Ranaviruses may have in fact been a cause of mortality in wild amphibian populations before we realized their importance in the last twenty years. Wolf *et al.* (1968) reported disease in wild bullfrog, *Rana catesbeiana*, tadpoles from West Virginia, USA, associated with a "polyhedral cytoplasmic virus" that he named Tadpole Edema Virus (TEV). TEV is morphologically similar to members of the Iridoviridae family and, when experimentally inoculated in various amphibian species, causes lesions most often associated with an infection with a *Ranavirus* sp: widespread visceral and epithelial necrosis and hemorrhage (Wolf *et al.*, 1968).

The first reports of mortalities due to a *Ranavirus* sp in Canada date back to the 1990s and occurred in tiger salamanders, *Ambystoma tigrinum diaboli*, from southern Saskatchewan (Bollinger *et al.*, 1999). The isolated virus was tentatively named Regina Ranavirus (RRV) and is now recognized as the Ranavirus species *Ambystoma triginum* virus (ATV) (Bollinger *et al.*, 1999; King 2011). Since then, reports of mortalities in wild populations of Canadian amphibians due to ATV, FV3 or FV3-like viruses have involved Sonora tiger salamanders (*Ambystoma mavortium*), Eastern grey tree frogs (*Hyla versicolor*), spring peepers (*Pseudacris crucifer*), green frogs (*Rana* [*Lithobates*] *clamitans*), Northern leopard frogs (*Rana* [*Lithobates*] *pipiens*), wood frogs (*Rana sylvatica* or *Lithobates sylvaticus*) and Eastern newt (*Notophthalmus viridescens*) (summarized in Miller *et al.*, 2011). A further 44 species have been affected by ranavirus-related mortalities in the rest of North America (Miller *et al.*, 2011). In comparison, and with a few notable exceptions, chytridiomycosis rarely causes severe mortalities in native North American amphibians. Severe declines have been observed in populations of Southern yellow-legged frog (*Rana muscosa*), for instance (Wake & Vredenburg, 2008), but native frogs in Canada, as those in the Eastern USA, have suffered no visible die-offs despite relatively high levels of infection with the chytrid fungus (Forzán *et al.*, 2010; Longcore *et al.*, 2007).

Although ranavirus- associated mortalities have not been tied to the decline of any North American amphibian population the way chytridiomycosis was demonstrated to have caused extinctions in Central American and Australian frogs, ecological disease modeling hypothesizes that extirpation of an isolated population of a highly susceptible species (wood frog) could occur as quickly as five years after introduction of a *Ranavirus* sp (Earl & Gray, 2014). The recognized importance of ranaviruses as emerging pathogens of cold-blooded vertebrates has resulted, amongst other initiatives, in the formation of a Global Ranavirus Consortium and the organization of biannual international meetings. Work presented at the three symposia convened so far has included field observational studies, mathematical modeling of disease ecology and experimental infection trials. While the significance of work on non-native species, specifically the African clawed frog (*Xenopus laevis*), is undeniable (i.e. Gantress *et al.*, 2003; Robert *et al.*, 2007; Robert *et al.*, 2011), scientists attending the First International Symposium on Ranaviruses emphasized the importance of conducting research on native species when studying the effect of ranaviruses on amphibians (Lesbarrères *et al.*, 2012).

Experimental trials, key to understanding the pathogenesis and host-pathogen interactions in ranaviral infections, have involved both native and non-native species but, with the exception of work on European common frogs (*Rana temporaria*) (Cunningham *et al.*, 2007), studies conducted on native amphibians have been restricted to the pre-metamorphic (tadpole) stage. If the immune system of tadpoles and adults in native species varies as it does in the laboratory frog *X. laevis* (Robert & Ohta, 2009), then a model of ranaviral infection in adult amphibians native to North American was lacking. The overall objective of the present work is to establish the wood frog as such a model. Wood frogs were chosen because they are widely spread across Canada and the rest of North America, reaching the farthest north in the continent, and have a high susceptibility to ranaviral infection (Duellman & Trueb, 1994; Lesbarrères *et al.*, 2012). Using the type species of the *Ranavirus* genus, FV3, for the experimental infections allows for comparisons with what is known to occur in *X. laevis* and provides information on the pathogenesis of the infection in the species most frequently involved in natural mortalities (Chinchar, 2002).

The specific objectives of this work, as developed in the following Chapters, are to:

I. Determine the husbandry requirements to successfully raise wood frogs in captivity from eggs to adults.

II. Establish the lethal dose 50 of FV3 for adult wood frogs, describe the clinical signs of infection, and determine the effect of dosage on the length of incubation period, lesions, median survival time and odds of death.

III. Review and summarize the scientific literature on clinical pathology of amphibians.

IV. Establish hematological reference intervals for adult wood frogs and determine the effect of FV3 infection on haematological analytes.

V. Elucidate the pathogenesis of fatal FV3 infection in adult wood frogs by evaluating the viability of fecal-oral transmission, describing the progression of lesions from time of infection to death, and determining the organs or tissues that act as important foci of viral replication and shedding.

In an effort to avoid excessive redundancy, and since the work for each individual objective is intended for (or has been published in) a peer-reviewed publication, each Chapter is headed by a detailed Introduction specifically relevant to its topic and objectives.

CHAPTER ONE

Notes on the captive husbandry of the wood frog, *Rana sylvatica* (*Lithobates sylvaticus*), an experimental animal model representative of North American native frogs

Abstract

Wood frogs, *Rana sylvatica*, are often used in ecotoxicological studies and have been proposed as a research model to study diseases of native amphibians in Native North America, but information regarding their captive husbandry is anecdotal and scarce. We describe the housing, feeding and veterinary care of a group of wild-caught wood frogs collected from urban and rural vernal pools and maintained in captivity for one year. Frogs collected as adults were housed in small groups (2-7 individuals) in polycarbonate cages lined with an unbleached paper towel, provided with water in Petri dishes and fed a combination of crickets, meal worms and earth worms. Tadpoles were housed in polycarbonate tanks and fed fish flakes and boiled lettuce; upon emergence of the limbs (metamorphosis), the froglets were transferred to paper towel-lined cages and introduced to live prey of an increasing size until feeding resembled that of adults. Survival in both groups was relatively good for wild-caught individuals: 75 % for wild-caught adults and 77 % for froglet to adults. Infectious diseases included cutaneous saprolegniasis in tapoles, which was successfully treated by adding 1-2 ppt of table salt in water for nine consecutive days, and pulmonary rhabdiasis in wild-caught adults, which was probably responsible for some mortalities but was controlled with 0.02 mg of Ivermectin/frog applied topically for three treatments six days apart and by avoiding the use of organic matter for bedding. The captive housing and rearing of wood frogs was labor intensive, and the commercial acquisition of live prey for feeding was costly. But after a routine for feeding, cleaning and health monitoring was developed, it proved practicable. Successfully maintaining wood frogs at all stages of development is crucial if this species is to become one of the main experimental subjects in the study of amphibian disease and host-pathogen interactions.

Keywords: Captive husbandry, *Rana sylvatica*, pulmonary rhabdiasis, saprolegniasis, wood frog

Introduction

Wood frogs (LeConte, 1825), *Rana sylvatica* or *Lithobates sylvaticus*, are members of the large Ranidae family. Ranid frogs, also known as “true frogs” as they fit the most recognizable body type associated with jumping amphibians, are found throughout the world except for southern South America, the West Indies, Australia and most oceanic islands (Duellman & Trueb, 1994). When first described by Captain John LeConte as “light to dark brown, with two interrupted longitudinal lines of black, a dark brown stripe extending from the tip of the nose through the eyes, and covering the auricles” wood frogs were given the scientific name of *Rana sylvatica* (LeConte, 1825). A revision of the amphibian nomenclature in 2006 (Frost *et al.*, 2006) proposed a switch from the genus *Rana* to *Lithobates* and adjusted the species’ Latin for “amidst the trees” accordingly (from *sylvatica* to *sylvaticus*).

Wood frogs are the most widespread of all North American frogs, extending across Canada, far into the shrubby tundra, and the eastern third of the United States. Adults prefer to live in closed-canopy deciduous and boreal forests and to reproduce in shallow, often temporal, bodies of water during early spring (Dodd, 2013). Partly because of their wide distribution and sympatry with many other amphibian species, wood frogs have been proposed as good experimental models for the study of infectious disease, particularly ranavirosis (Lesbarrères *et al.*, 2012). Ranaviruses, members of the Iridoviridae family of double-stranded DNA viruses, are emerging pathogens of cold-blooded vertebrates (Chinchar, 2002) and the focus of intense field and laboratory research (Chinchar & Waltzek, 2014). Unfortunately, except for some anecdotal reports or broad guidelines referring to amphibians in general (e.g. Canadian Council for Animal Care, 2004; Wright & Whitaker, 2001) there is little to no information on the husbandry and feeding of wood frogs in captivity. Laboratory research on, and experimental infections with, amphibian ranaviruses have focused mainly on non-native species, specifically the African clawed frog (*Xenopus laevis*) or pre-metamorphic (tadpole) stages of native species including the wood frog (e.g. Gantress *et al.*, 2003; Hoverman *et al.*, 2010). One of the reasons for the paucity of studies involving captive adult wood frogs is their feeding: whereas wood frog tadpoles can be fed easily with various insect larvae or commercial pellets or flakes (Wright & Whitaker, 2001), adult wood frogs require live prey. Additionally, because there are no commercial sources of wood frogs, all experimental animals (be it eggs, tadpoles or adults) must be wild-caught. Wild animals may be already infected with the pathogen of interest or, even when free of a specific pathogen, may introduce other unwanted pathogens to the captive facility.

Despite the disadvantages of working with wild-caught individuals for which care there is little guidance, studies conducted on wood frogs are highly relevant to the understanding of diseases in wild populations. Findings on the pathogenesis and host-pathogen interaction of wood frogs experimentaly infected with ranaviruses may be more directly applicable to wild native North American frogs than those obtained from studies in non-native species, for instance.

Our objective is to describe the successful husbandry, feeding and veterinary care of wild-caught wood frog tadpoles, recent metamorphs (froglets) and adults during a year in captivity.

Materials and Methods

Representative images of the captive frogs are included in Figure 1.

Origin of wood frogs

Wood frog adults (n=59) and several egg masses were collected between April 17th and 21th, 2012, from two vernal pools in Prince Edward Island. Most frogs were caught in an urban/suburban pool (n=56 frogs, N 46.24701 W 63.12828), only a few were caught in a rural pool (n=3 frogs, N 46.25543 W 62.71187). Egg masses, and later tadpoles, were collected from the urban/suburban pool on April 29th and May 17th respectively. Methods for collection and housing followed a protocol approved by the Animal Care Committee of the University of Prince Edward Island (UPEI, 12-014, 6004702). Husbandry practices were in part based on recommendations from Dr Paula Jackman (Environment Canada, Moncton, New Brunswick) and Dr Todd Smith (Acadia University, Wolfville, Nova Scotia), both experienced in captive rearing of native amphibian species.

Eggs and tadpoles

On April 29th, a few days after collecting the adults, several egg masses were collected in approximately 5 L of pond water from the same urban/suburban vernal pool (46.24701 W 63.128283). Several tadpoles hatched in the collected water, immediately upon collection. The eggs and hatched tadpoles were allowed to acclimate to the temperature of the room in which they were to be housed (NB144, Atlantic Veterinary College, North Barn). Two hours later, they were placed in a polycarbonate tank (38 x 47 cm, holding approximately 25 L of water) filled with 50% pond water and 50% dechlorinated tap water that had been passively aerated in open containers for a minimum of 24 hours. An airing stone was placed in the tank and small amounts of dechlorinated water were added everyday as more tadpoles hatched. When commercial fish flakes (Tetra, United Pet Group) were first offered on the third day of captivity (May 1st), none of the tadpoles was interested. A few tadpoles (3-5) were finally seen feeding on boiled lettuce offered two days later (May 3rd). Small amounts of fish flakes and lettuce were offered daily from then on and tadpoles ate *ad libitum*. Between the seventh and tenth days of captivity (May 5th-8th) the tadpoles were split into three other tanks by transferring water from the original tank and adding dechlorinated water and letting it sit for 24 hours before moving tadpoles in. When it was clear that simply adding water would not be sufficient to keep the tanks from fowling, syphoning of debris at the bottom was performed (May 9th). Unfortunately, mortalities began on day 14 of captivity (May 12th) and, in spite of aggressive water changes and removal of debris, all tadpoles were dead by day 18 of captivity (May 16th). Histopathological examination revealed no evidence of an infectious etiology; the tentative diagnosis was ammonia toxicity.

A drastic change in our approach was clearly needed. Since egg masses were no longer present, a second batch of approximately 150 tadpoles was collected from the same vernal pool on May 17th. The tadpoles were placed in four tanks, each with approximately 30-40 tadpoles, in a similar combination of pond water to clean water (50/50) but, instead of the more natural habitat we originally intended by only adding small amounts of clean water, changes of large volumens of water were performed daily. Each day, from 50-80% of the tank water was syphoned from each of the four tanks and slowly replaced with clean well water. Aerated tap water was no longer used. Additionally, ammonia was measured daily with an aquarium kit and, later on, Ammonia Alert disks (Seachem Laboratories, 1000 Seachem Drive, Madison, GA 30650) were placed on the side of the tank for continuous monitoring. Ammonia levels were maintained at approximately 0.05 ppm (mg/L), as per Ammonia Alert disk measurements. Approximately two weeks later, water changes became less aggressive, and only 30-50% of the volume was replaced daily. More aggressive changes were only performed if ammonia reached 0.2 ppm (mg/L). Daily water changes and ammonia monitoring were performed daily until all tadpoles had completed metamorphosis (from May 17th to August 10th). Feeding continued *ad libitium*. Each day remnants of fish flakes or boiled lettuce from the previous day were removed (either with a net or during the daily syphoning) and fresh food was added.

Any obviously ill (*i.e.* floating upside down or circling) or dead tadpole observed during daily cleaning and feeding was immediately removed. If alive, tadpoles were euthanized by immersion in 70% ethanol (Wright & Whitaker, 2001). A few of the mortalities (approximately 20) were preserved in 70 % ethanol in case they were needed to rule out an infectious disease.

Upon emergence of back legs (first observed 30 days after collection, on June 18th), individual tadpoles were moved to a transition tank with a lower water level and plastic plants to climb onto. Once all four legs were present and a short tail remained, each recent metamorph (froglet) was moved to one of seven dry cages (8-14 frogs per cage) with shallow water (Petri) dishes on an unbleached paper towel. The first froglet was moved to a dry tank six days after the back legs were noticed (June 24th). Five days after the last of the froglets was moved to a dry tank (August 5th), the remaining five tadpoles (which had failed to undergo metamorphosis) were euthanized in 70% ethanol.

Froglets (captive-raised metamorphs)

Close to seven weeks elapsed between the move of the first and last froglets to the dry cages (June 24th - August 10th). Aged (dechlorinated) tap water was provided in Petri dishes, one to two per cage. Froglets were fed increasingly larger insects as they grew. When first placed in the dry tanks, wingless flies (*Drosophila melanogaster*) approximately 2.5 mm long were fed two or three times a day. On July 21st, very small mealworms (*Tenebrio molitor*) less than 5 mm, were added to the diet. Pinhead (recently hatched) and 1/16-inch crickets (*Acheta domesticus*) less than 2 mm long began being offered on July 25th. Larger flightless flies (*Drosophila hydae*) approximately 3.5 mm long were offered from August 3rd onwards. Small wild-caught earth worms, less than 10 mm long began being offered on August 8th. At least one of the daily feedings consisted of wingless flies dusted in a 50:50 mixture of calcium carbonate and multivitamin powder (Reptivite, Zoo Med Laboratories Inc., San Luis Obispo, California, USA).

A linear fluorescent bulb of UVB light (Repit-Glo 2.0, Exo-Terra, Rolf C. Hagen Inc.) was placed just above the gridded cover of the cages, 30-35 cm from the bottom, and turned on automatically for a 12-hr light/dark cycle that matched the original light cycle in the room (fluorescent lamps); the UVB light exposure began (September 2nd) approximately two months after the first froglets had completed metamorphosis and continued through early adulthood to the end of their captivity at AVC on May 29th 2013.

Originally housed in the same room as the wild-caught adults, froglets were moved in early autumn (October 16th) to a separate room (NB122) where humidity could be raised above ambient levels.

All mortalities were recorded.

Wild-caught adults

Adults were housed in polycarbonate cages in groups of three to seven individuals (room NB144). Groups remained unaltered unless an individual died, had to be euthanized, or was isolated. Isolation could be due to hyperactivity or depression (assumed to represent stress from cohabitation or mild illness, respectively). Isolated frogs were housed individually. The three individuals from the rural vernal pool were housed together and always cleaned and fed last.

For the first five weeks, the floor of the cages was lined with the commercial terrarium substrate Forest Moss (Exo-Terra, Rolf C. Hagen Inc.) but on June 21st a switch to bleach-free paper towel was made. Unbleached cardboard tubes cut longitudinally in half provided hiding spots. Aged (dechlorinated) tap water was provided in Petri dishes, one to two per cage.

Feeding consisted of mealworms, wild-caught earthworms and medium to large (15-25 mm long) crickets. A schedule of four mealworms, three earth worms and three crickets per week per frog was used as a baseline but adapted to suit changes in appetite. Crickets dusted with the same 50:50 mixture of calcium carbonate and multivitamins that was used to dust the fruit flies fed to froglets were offered at least once a week. The number of prey items fed per cage was recorded, as was the number of prey that remained uneaten and dead prey that were removed.

All mortalities were recorded and carcasses examined grossly, fixed in 70% ethanol or 10% formalin and examined histologically.

Feeding sources: commercial and produced in-house

The commercial fish flakes (Tetra, United Pet Group) fed to the tadpoles were purchased from a local pet shop (Critters, Kent Street, Confederation Court Mall, Charlottetown, PEI). The lettuce was purchased from a local supermarket (Atlantic Superstore, University Avenue, Charlottetown, PEI). A source colony of wingless *Drosophila melanogaster* fruit flies was donated by the Environment Canada Laboratory, University of Moncton, and corresponded to WARD'S Live Drosophila Culture, Apterous [Wingless, Recessive], catalog number 876565 (WARD'S Science, Vansickle Road, St. Catharines, Ontario, Canada). The source colony of flightless *Drosophila hydae* fruit flies was purchased from RECORP Inc. (Georgetown, Ontario, Canada). Mealworms were purchased from the local pet shop (Critters) and grown to adults to establish an in-house breeding colony. Except for a few crickets that had been raised at the North Barn of the AVC and donated to the wood frog room, crickets were initially purchased from the local pet shop (Critters) and, when the numbers required surpassed the shop's stock, mail-ordered from a commercial breeder (Super Cricket, Henribourg, Saskatchewan, Canada).

Colonies of *D. melanogaster* and *D. hydae* were grown in-house using commercial fruit fly media (WARD'S Instant Drosophila Medium, White, Canada). Mealworms were grown in a 50:50 mixture of bran and wheat flour (Bulk Barn, University Avenue, Charlottetown) supplemented with potato or fruit slices.

Light/dark cycle

Automatic ceiling lights in the room housing wild-caught adults and tadpoles/froglets (NB144) were on from 0630 to 2200 (15.5/8.5 hr light/dark cycle) between April 17th and September 19th. The light period was decreased slowly (by one hour every two weeks) until room lights were on from 0630 to 1830 (12/12 hr light/dark cycle) by October 14th.

Linear fluorescent bulbs of UVB light (Repit-Glo 2.0, Exo-Terra, Rolf C. Hagen Inc.) were placed directly on top of the lidded cages housing froglets on September 2nd. Froglets were exposed to UVB light for at least 10.5 hr/day. When froglets were moved to a separate room (October 16th), ceiling lights were only turned on when needed for cleaning and feeding. The UVB lamps served as primary light sources and followed the 12/12 hr light/dark cycle (0600 to 1800) for approximately 105 days (September 2nd to December 13th). To reflect the seasonal patterns, the cycle was changed to 10/14 hr light/dark (0700-1700) on December 14th, 8/16 hr light/dark (0730-1630) on December 30th, 10/14 hr light/dark (0730-1730) on March 13th 2013, 11/13 hr light/dark (0700-1800) on April 6th, and back to a 12/12 hr light/dark cycle on April 14th. The 12/12 hr light/dark cycle was maintained until May 29th 2013 when the last of the froglets, by then adults, were transferred out of the AVC.

Temperature and humidity

The temperature and humidity of the rooms where the animals were housed (NB144 for wild-caught adults and tadpoles ; NB122 for froglets and adults raised from wild-caught tadpoles) were recorded daily, both as the value read immediately after entering the room in the morning, and as the minimum and maximum values of the previous 24 hours.

Cleaning and disinfecting

Working form the assumption that wild-caught animals were potential carriers of known and unknown pathogens, the room where the wild-caught adult frogs, eggs and tadpoles were housed (NB144) was subdivided into a dirty area (where the animals were housed) and a clean area where the prey was raised and any materials were stored. Stainless steel shelves were placed in a way to provide a barrier between areas, leaving only a 50-cm-wide communicating space between them. Only designated slip on plastic shoes were worn in the dirty area; they were placed at its entry to allow for an easy switch. Cleaning material, including labcoats, latex gloves and spray bottles, were designated for each side of the room and, when possible, color-coded to avoid confusion if an item was accidentally moved from one area to the other. A 5% bleach solution (5 parts of 10% sodium hypochlorite:95 parts tapwater) (CDC & WHO, 1998) was used to disinfect any material that had come in contact with a frog or tadpole. All materials were spayed with the bleach solution in the dirty area and placed on a designated transfer stainless steel shelf to sit for at least 10 minutes before entering the clean area; once in the clean area, they were re-spayed with bleach, washed with dish detergent and rinsed. Clean materials were then placed on a separate designated shelf (above the one used for the dirty material) to dry and be available for used in the dirty area.

Cleaning began with the tadpole tanks (or froglets cages later on) and ended with the wild-caught adults if done by one person. If there were two people, one usually concentrated on tadpoles/froglets while the other cleaned the adults. Tadpole and froglet enclosures were cleaned on the shelf and table where they were placed, respectively. Wild-caught adult cages, as they were stacked on shelves, had to be individually moved to a designated handling (dirty) table, where they would be cleaned (*i.e.* change paper towel, replenish or replace water dish, remove soaked cardboard hiding spots and dead prey). Paper towels were usually changed daily. When the cage had become too dirty or the paper towel was soaked, a new cage was prepared and the frogs would be moved to it. The dirty cage would be sprayed with bleach and left aside for North Barn personnel to remove and take to the automated cage washing facility. After cleaning, the frogs were fed and then the cage placed back on the shelf. After each cage was cleaned, the gloves were sprayed with bleach and, after a few minutes, sprayed with tap water to rinse them before handling the next cage or frog. At the end of the cleaning process, the table was sprayed with bleach, rinsed and dried. All used paper towels, cardboard and dead prey were collected in a plastic container and later incinerated.

Veterinary care

Two infectious diseases presented in the captive wood frogs and required medical intervention: a protistan (or water mould) infection in tadpoles and an intense parasitic infection in wild-caught adults.

On May 23rd, after a velvety growth had been observed on the skin of a few tadpoles in one of the tanks and diagnosed clinically (visual inspection) as infection with the oomycete or water mould *Saprolegnia* sp, table salt (NaCl) was added to the water at 2 ppt (2 g/L). Initially the salty water bath was tried on a small group of tadpoles; whole tanks were only treated once it was evident that the salinity caused no ill effects. Within two days of treatment, only rare tadpoles with the velvety growth could be seen, so the concentration of salt was reduced to 1 ppt (1 g/L). After 9 days of treatment (June 1st), the salt was completely discontinued. No further *Saprolegnia* sp infections occurred.

A heavy infection with pulmonary nematodes, *Rhabdias* sp, was diagnosed on histopathology in some adults within the first month of captivity. All adult frogs were treated with a dose of 2 mg/kg of Ivermectin (0.02 mg of Ivermectin/frog) applied topically on the dorsal skin. Three treatments were applied: May 19th, May 25th and June 1st. Treatments stopped after fecal examinations (Baerman technique) were negative for lungworm larvae.

Results

From the second batch of approximately 150 tadpoles collected, 118 succesfully completed metamorphosis. A few tadpoles (exact number was not recorded) were accidentaly killed during tank cleaning when succioned along with debris resulted in their evisceration. Occasionally, tadpoles were observed feeding on a carcass. Wood frogs are explosive breeders and all egg laying occurs within a period of a couple of days. Therefore, assuming all tadpoles hatched at approximately the same time, the first signs of metamorphosis, namely the emergence of the back legs, occured approximately day 45 post-hatching. Counting from the time the first sign of metamorphosis was observed to the time the first froglet was placed in a dry tank, metamorphosis lasted approximately a week; complete resorption of the tail required a further two to three days.

Of the 118 froglets that completed metamorphosis, 27 died during the first 75 days after the last one completed metamorphosis (last metamorphosis completed on August 10th, last mortality occurred on October 19th 2012). Afterward, all 91 froglets survived until adulthood, a little over a year after hatching, when their captivity at AVC ended and they were transferred to other facilities. The majority of the mortalities (19/27) occurred during the first 30 days after completion of metamorphosis (from August 10th to September 7th). Mortalities slowed down considerably within a week of adding the UVB lights over the froglet cages. Overall survival from froglet raised from tadpole to captive-raised adult was 77 %.

Survival of wild-caught adults was 75 % (44/59), most mortalities (14/15) occurred during the first 60 days of captivity (penultimate mortality June 9th 2012). The last mortality, which occurred five months into the captivity period (September 13th) was that of a frog that had been isolated 30 days after collection due to its weakly demeanor. Of the 21 frogs placed in isolation, eight died; the remaining 13 survived until the end of captivity at the AVC.

Temperature and humidity in the room where the wild-caught adults and tadpoles/early froglets were housed (NB144) averaged 21°C (average min-max 20-22°C, median 21°C, min-max 18-23°C) and 34 % (average min-max 28-44 %, median 36 %, min-max 10-70 %), respectively, from August 3rd to October 15th. Temperature and humidity in the room where the froglets/captive-raised adults were housed (NB122) averaged 21°C (average min-max 21-22°C, median 21°C, min-max 19-21°C) and 52 % (average min-max 46-62 %, median 52 %, min-max 13-74 %), respectively, from October 12th to May 28th.

Discussion

Survival in both the froglet/captive-raised adults (from metamorphosis to one-year adulthood) and wild-caught adults was fairly high (77 % and 75 %, respectively). The exact number of tadpoles that died between collection and the end of metamorphosis is unknown. It was impossible to accurately count the tadpoles that were collected and, although some mortalities were preserved to rule out infectious diseases, not all mortalities (accidents during cleaning and cannibalized carcasses) were recorded. Even if every dead individual found in the tanks had been reported, because of the cannibalistic habits of tadpoles (Duellman & Trueb, 1994), it is likely that many dead ones were consumed before they could be removed or counted. An alternative to the time-consuming daily water changes would have been to place the tadpoles in a stable environment with charcoal filters and re-circulating water. Such a setting involves planning and time. Establishing a viable bacterial population in a tank, for instance, requires several weeks. Lack of previous experience in tadpole rearing and the necessity to work within the constraints of the material at hand meant that we did not consider a more sophisticated but less labour-intensive set up until the tadpoles had already been collected – much too late to set it up.

Froglet growth was remarkably better than that observed in the same species at the Environment Canada laboratory in Moncton (as assessed by Dr Paula Jackman in a visit to AVC in November 2012). High survivability required a substantial time commitment and a relatively high cost. During most of the year that the frogs were housed at the AVC, their care required from 3-8 person-hours at least six days a week. The insects used for feeding were another drain on resources. Purchasing live prey items was expensive. In-house growing of live prey, while less expensive, was labor intensive.

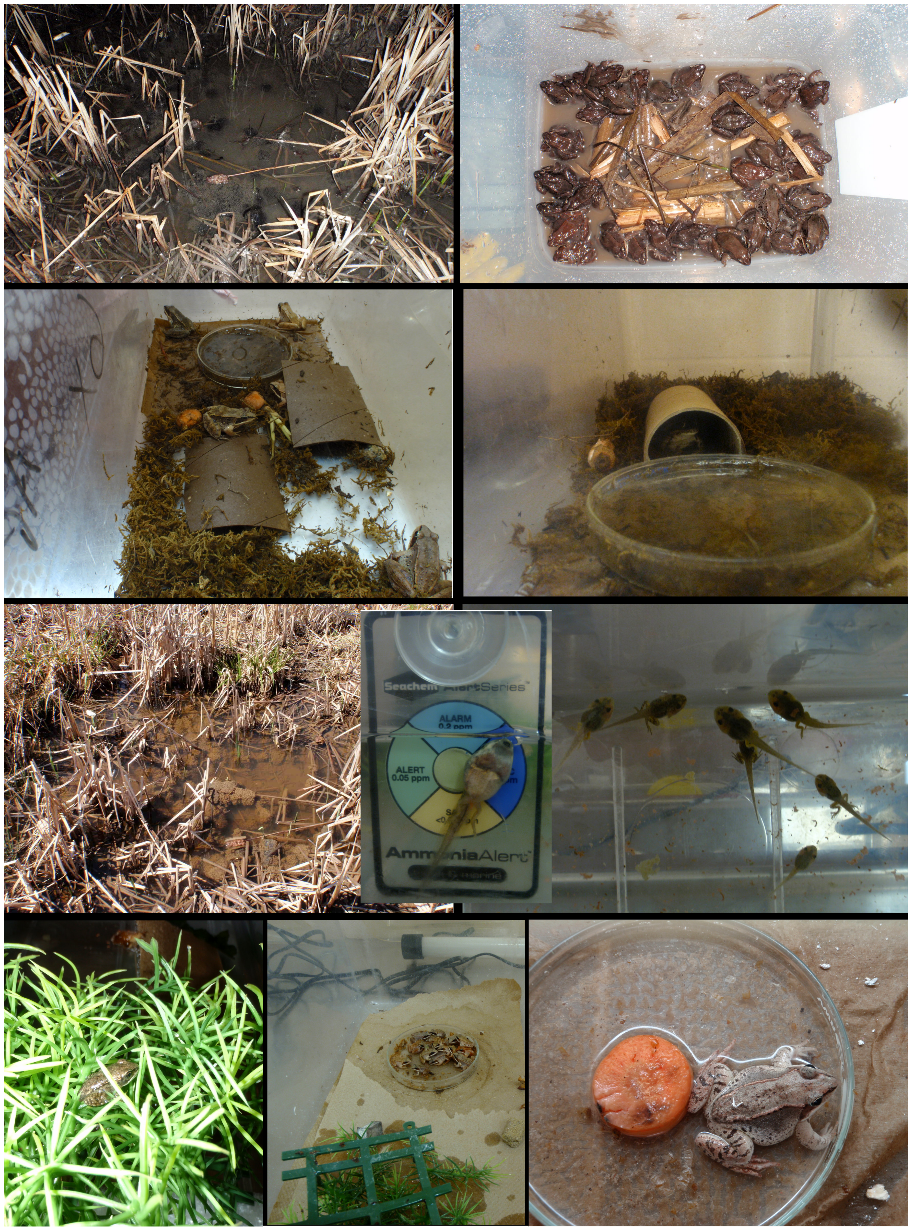
Salt was a gentle, well-tolerated and effective treatment for saprolegniosis in tadpoles. The treatment of Ivermectin along with the subsequent lack of positive fecal samples and the change from moss to unbleached paper towel as substrate in the cages eliminated the lungworm infection in the colony. Originally, we intended to collect skin swabs to test for the presence of *Batrachochytrium dendrobatitidis*, the fungus responsible for chytridiomycosis that has been reported in wild populations on Prince Edward Island (Forzán *et al.*, 2010). As the first mortalities occurred, however, and no evidence of infection was found in any of the dead frogs, swabbing was deemed unnecessary.

The captive housing and rearing of wood frogs was labor intensive and expensive but, after a routine for feeding, cleaning and health monitoring was developed, it proved practicable. Successfully maintaining wood frogs at all stages of development is crucial if this species is to become one of the main experimental subjects in the study of amphibian diseases and host-pathogen interactions.

Acknowledgments

This work required the help of numerous people who were involved in the capture of the frogs, helped out with their rearing, generously donated breeding stock for the colonies of fruit flies, or provided invaluable suggestions regarding husbandry, feeding and veterinary care. In no particular order, my thanks to: Raphaël Vanderstichel, Shannon Martinson, Sara Vazquez Quiroga, Jessica Thompson, Sophie St-Hilaire, Dawson Chalmers, Jonathan Spears, Marion Desmarchelier, Marianne Parent, Wayne Petley, Paula Jackman and Todd Smith.

Figure I - 1. Wild origin and captive housing of wood frogs, *Rana sylvatica*. From top left to bottom right: Adults floating in urban pond on the night that most were collected (April 21, 2012). Collected adults in plastic container used for transport to the Atlantic Veterinary College (AVC). Adults housed in polycarbonate tank lined with moss. Adult frog hiding under cardboard tube next to water-filled Petri dish. Egg masses in urban pond. Tadpole in front of ammonia measuring disk. Tadpoles after hind legs have emerged. Recent metamorph (froglet) that had climbed the only plastic plant in transition tank. Group of froglets in Petri dish a few days after being transfer to a dry tank. Adult wood frog in Petri water dish along side a slice of carrot.



CHAPTER TWO

Clinical signs, pathology and dose-dependent survival of adult wood frogs, *Rana sylvatica*, inoculated orally with Frog Virus 3 (*Ranavirus* sp, Iridoviridae)[1]

Abstract

Amphibian populations suffer massive mortalities from infection with Frog Virus 3 (FV3, Ranavirus, Iridoviridae), a pathogen also involved in mortalities of fish and reptiles. Experimental oral infection with FV3 in captive-raised adult wood frogs, *Rana sylvatica* [*Lithobates sylvaticus*], was performed as the first step in establishing a native North American animal model of ranaviral disease to study pathogenesis and host-response. Oral dosing was successful; the LD_{50} was $10^{2.93\ (2.42\text{-}3.44)}$ pfu for frogs averaging 35 mm in length. Onset of clinical signs occurred 6-14 days post-infection (dpi) (median 11 dpi) and time-to-death 10-14 dpi (median 12 dpi). Each ten-fold increase in virus dose increased the odds of dying by 23-fold and accelerated onset of clinical signs and death by approximately 15%. Ranavirus DNA was demonstrated in skin and liver of all frogs that died or were euthanized because of severe clinical signs. Shedding of virus occurred in feces (7-10 dpi; 3-4.5 d before death) and skin sheds (10 dpi; 0-1.5 d before death) of some frogs dead from infection. Most common lesions were dermal erosion and hemorrhages, hematopoietic necrosis in bone marrow, kidney, spleen and liver, necrosis in renal glomeruli and in tongue, gastrointestinal tract, and urinary bladder mucosa. Presence of ranavirus in lesions was confirmed by immunohistochemistry. Intracytoplasmic inclusion bodies (probably viral) were present in the bone marrow and the epithelia of the oral cavity, gastrointestinal tract, renal tubules and urinary bladder.

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[1] MJ Forzán, KM Jones, RV Vanderstichel, J Wood, FSB Kibenge, T Kuiken, W Wirth, E Ariel, P-Y Daoust. Journal of General Viology, 2015;96:1138-1149. Adapted and expanded version of the published manuscript.

Our work describes a Ranavirus-wood frog model and provides estimates that can be incorporated into ranavirus disease ecology models.

Keywords: Frog Virus 3; LD_{50}; *Lithobates sylvaticus*; *Rana sylvatica*; ranavirus

Introduction

Frog Virus 3 (FV3), the type species of the genus *Ranavirus* (family Iridoviridae), was isolated more than 50 years ago (Granoff *et al.*, 1966) but not until the beginning of the 1990s was it recognized as the pathogen responsible for high mortality epizootics in fish, amphibians and reptiles (Chinchar *et al.*, 2009; Lesbarrères *et al.*, 2012). In 2008 infection with *Ranavirus* sp became one of only two notifiable diseases of amphibians listed by the World Organisation for Animal Health (OIE, 2013). Ranaviruses have been responsible for mass mortalities in wild and captive frogs and salamanders in North America, Asia, Australia and Europe (Gray *et al.*, 2009), and are currently the focus of intense research (Chinchar *et al.*, 2009). Experimental infections with various species and isolates of ranaviruses have been achieved through intraperitoneal injection (eg Tweedel & Granoff, 1968; Wolf *et al.*, 1968), immersion in viral suspension via water bath (eg Brunner *et al.*, 2005; Harp & Petranka, 2006; Cullen & Owens, 2002), exposure of cutaneous wounds to virus (Cunningham *et al.*, 2007) and oral administration (Wolf *et al.*, 1968; Hoverman *et al.*, 2010). Although the work of dozens of researchers, past and present, frequently focuses on experimental challenges with the original FV3 isolate (Granoff 1965), reported dosages vary, as do the species and developmental stage of the infected host. Research on FV3 is particularly relevant since many mortality events throughout the world are due to FV3 or FV3-like viruses (Chinchar, 2002). Similarly to other viruses, the dose and route of infection are important determinants of FV3 pathogenicity (virulence, type and severity of lesions) (Brunner *et al.*, 2005; Cullen & Owens, 2002; Cunningham *et al.*, 2007). Thus, the variability in experimental designs provides an abundance of valuable information but complicates comparisons and extrapolations. Amongst the multiple host species used in research, it is arguably in the African clawed frog, *Xenopus laevis*, that the host response of adult frogs to, and pathogenesis of, FV3 infection have been most extensively studied (Gantress *et al.*, 2003; Robert *et al.*, 2007; Robert *et al.*, 2011). Adult *X. laevis* inoculated intraperitoneally with $10^{7.7}$ pfu of FV3 show only transitory signs of disease that are correlated with the presence of viral DNA in the kidney; signs disappear 2 w post-infection while virus becomes undetectable in most tissues 1 m post-infection when specific anti-FV3 IgY antibody production peaks (Gantress *et al.*, 2003) although it may remain present in the kidney for several months (Robert *et al.*, 2007) and possibly result in excretion via urine-rich feces (Gantress *et al.*, 2003). Unfortunately, *X. laevis* is a member of a family of frogs (*Pipidae*) not naturally present in North America or the rest of the northern hemisphere, which limits its regional relevance to the study of disease ecology in native amphibians. The anatomy and natural history of pipid frogs, which are restricted to tropical South America east of the Andes and to sub-Saharan Africa, differ significantly from those of frogs of the northern hemisphere: pipids are strictly aquatic and thus morphologically adapted to this environment with fully webbed feet, lateral-line organs, poorly developed to absent eyelids, no tongue and a diet composed mostly of zooplankton (Duellman & Trueb, 1994). The validity of extrapolating findings on pipids to native North American frogs, particularly regarding mode of transmission, carrier states and pathophysiology, should be questioned since phylogeny, life history and type of habitat have been suggested to influence host susceptibility and response to ranavirus infection (Hoverman *et al.*, 2011). A better representative of the life history of the majority of frogs in the northern hemisphere and of those North American species in which the majority of mortalities have been reported since 1997 (Hoverman *et al.*, 2011) is the family Ranidae, or true frogs. Ranid frogs are present across the entire northern hemisphere and extend the farthest north of any other amphibian species. The ranid anatomy, life history and reproductive strategy are those of the archetypical frog: mostly terrestrial (riparian, fossorial or occasionally arboreal), with a carnivorous/insectivorous diet, external fertilization, and egg laying and larval development in water (Duellman & Trueb, 1994). Of the 29 North American species of ranids, at least 14 of which are known to be susceptible to ranavirus infection and disease (Miller *et al.*, 2011), the wood frog, *Rana sylvatica* [*Lithobates sylvaticus*], was proposed as a focus for research by participants at the First International Symposium on Ranaviruses (Lesbarrères *et al.*, 2012). Researchers at the Symposium emphasized the need for an amphibian model for viral challenge experiments that allows for comparisons amongst studies and provides data to incorporate into ecological disease models, and thus selected the wood frog given its life history, high susceptibility to disease caused by FV3 and widespread distribution in North America which makes it sympatric with many other native species (Lesbarrères *et al.*, 2012). Experimental infections in wood frog tadpoles suggest that the susceptibility of the species to infection and mortality due to FV3 and FV3-like viruses is similar to, or slightly higher than, that of other sympatric North American species (Hoverman *et al.*, 2011). To our knowledge, no reports exist of experimental infection of adult wood frogs. Establishing a useful model of FV3 infection in wood frogs will allow for comparisons with what is known to occur in *Xenopus laevis* and, more importantly, provide information on the pathogenesis of the infection in a species designated as a representative of frogs commonly affected by the disease.

One of the first steps in establishing a native North American animal model to study ranavirus pathogenesis and host response to infection is the determination of the dose of virus necessary to cause mortality in 50% of individuals: LD_{50}. A known LD_{50} allows for the design of virus challenge experiments with specific aims and for a meaningful comparison of results amongst studies. Along with a pre-determined dose, it is necessary to find a route of administration that can mimic transmission in the wild, is easily employed, and allows for the administration of precise dosages. As consumption of infected material (scavenging on infected carcasses) is a known route of ranavirus infection in wood frog tadpoles (Harp & Petranka, 2006) and since oral dosing allows for the administration of predetermined viral concentrations, the oral route likely fulfills those requirements.

Our overall aim was to propose parameters that can become the standard for future ranavirus-North American frog models. Our specific objectives were to establish the LD_{50} of FV3 virus in one-year-old captive-raised wood frogs when administered orally, determine parameters potentially useful in disease modeling such as length of incubation period, median survival time (ST_{50}) and odds of death at a certain viral dose, establish whether shedding occurs in feces and skin sheds and describe the clinico-pathological changes resulting from infection.

Materials and Methods

Origin and housing of experimental subjects. Wood frog tadpoles were collected from an urban pond in Prince Edward Island, Canada, 1-2 weeks after hatching (2012-05-17) and housed in accordance with guidelines of the Canadian Council on Animal Care (CCAC, 2004; Supplemental Figure S1a-b). Tadpoles (later frogs) were maintained at a fairly constant room temperature both before and during the experimental infection (overall average 21-22°C). Humidity varied considerably and reflected the seasonal ambient temperature (overall average 41-56%) (Supplemental Table S1). All mortalities (29/112 frogs that completed metamorphosis) that occurred in the months prior to the experiment were examined grossly and histologically for any lesions suggestive of a ranavirus infection since there is no reliable method to detect subclinical infection in live animals. As none of the mortalities had any histological evidence of a ranavirus infection, we assumed that the captive-raised animals were free of the virus. One year post-hatching (2013-05-02), the 34 frogs used in the experimental trial were placed in individual tanks and randomly assigned to an infection (n=30, 5 frogs/dose of inoculum) or control group (n=4). After six days of acclimation to their individual tanks, the frogs were inoculated with FV3.

Frog Virus 3 culture. The viral strain used in this study, originally isolated in 1965 from a Northern leopard frog, *Rana* [*Lithobates*] *pipiens* (Granoff *et al.*, 1966), had been grown sequentially by various researchers in a variety of cells: Fathead minnow cells (FHM cells, at least 15-25 passages, G. Chinchar personal communication), *Xenopus* A6 cells and Baby Hamster Kidney fibroblasts (1-2 and 2 passages, respectively, J. Robert personal communication) and *Epithelioma papulosum cyprini* (EPC) cells (2-3 passages, A. Reid personal communication). For these study, the stock was grown by the authors in EPC (1 passage) at room temperature (18-20°C). The isolate used in this study calculation of LD_{50} dose followed the Reed and Muench method (Reed & Muench, 1938) corroborated by a logistic regression (**Supplemental Figure S2)**.

(Supplementary Material). Titrations of viral stock were performed to determine median Tissue Culture Infective Dose ($TCID_{50}$) and plaque forming units following standard methods (Reed & Muench, 1938; Dulbecco & Vogt, 1953). Calculation of LD_{50} dose followed the Reed and Muench method (Reed & Muench, 1938) corroborated by a logistic regression.

Inoculation, termination and sample collection. On inoculation day, each frog was orally administered 50 µl of minimum maintenance medium (MEM supplemented with 2% fetal bovine serum and 1% Antibiotic Antimycotic, Invitrogen) containing 0 (control group, n=4), $10^{0.43}$, $10^{1.43}$, $10^{2.43}$, $10^{3.43}$, $10^{4.43}$ or $10^{5.43}$ pfu of FV3 (infection groups, n=5/dose of inoculum) through a graded pipette (Figure S1c). The small volume of inoculum was chosen to avoid any regurgitation. Frogs were checked two or three times daily to record specific clinical signs. Feces and skin sheds found in the water dish of control and inoculated frogs were collected opportunistically, frozen at -80°C and later tested for ranavirus DNA by PCR. Upon every collection the water dish was disinfected and refilled with clean water. All handling started with the controls and continued through the infection groups from lowest to highest virus dose; equipment (i.e. plastic gloves, metal forceps) was disinfected with sodium hypochlorite (5% bleach solution) after handling each frog or enclosure.

Euthanasia was performed at a pre-determined endpoint (when frogs exhibited signs indicative of serious illness [Wright & Whitaker, 2001] that would have eventually resulted in death) instead of allowing death to occur naturally. Thus, euthanasia (by immersion in a 10% solution of tricaine methanesulfonate [TMS, Syndel Laboratories LTD]) was performed when a frog exhibited two or more of the following signs: severely depressed appearance (head down and back legs extended with loss of normal upright posture and of withdrawal reflex), loss of righting reflex, or presence of many petechial haemorrhages in the skin of the fore or hind feet, inner thighs or ventrum (Figure 1a-1b). The experiment was terminated 22 dpi, eight days after the last mortality occurred, by euthanizing all remaining frogs. Previous studies have considered 21 dpi sufficient for infection and morbidity due to ranavirus to occur (Hoverman *et al.*, 2011).

Immediately after death the snout-vent (SV) length was measured, a necropsy performed, and gross lesions recorded. Weight, being extremely variable due to hydration status, food in the stomach and urine in the bladder (Wright & Whitaker, 2001), was intentionally not recorded. Samples of ventral skin and left liver lobe were collected, frozen at -80°C and later tested for ranavirus DNA by PCR. The rest of the carcass was preserved in 10% buffered formalin. The formalin-fixed carcasses of three frogs from each dose group and two control frogs were processed routinely for histologic examination (10 months post-fixation). Tissues, sectioned at 5 µ and stained with hematoxylin and eosin, included one fore foot, one hind foot, a median section of the head and jaw, a cross mid-shaft section of the thigh, and sections of heart, lungs, abdominal fat body, liver, kidneys, urinary bladder, stomach, intestine, colon, spleen and reproductive organs. All procedures followed a protocol approved by the Animal Care Committee of the University of Prince Edward Island.

Immunohistochemical staining. A subset of the tissues examined histologically was stained immunohistochemically using a primary antibody known to cross-react with FV3 (Ariel *et al.*, 2010) to detect the presence of viral particles. Briefly, 5-µ sections were deparaffinised by immersion in two separate baths of xylene (3 min each), three separate baths of 100% ethanol (2 min each), and rinsed in running tap water (1 min). After antigen epitope retrieval was achieved by boiling in TE (Tris/EDTA pH 8.5) solution for 20 min using an 850W microwave, the slides were washed with tap water, carefully dried, and a well was created around the tissue sections to hold the IHC solutions. Slides were washed three times with TE, blocked with ELISA buffer containing casein (30 min at room temperature) and incubated (1.5 h at room temperature) with 50 µl of rabbit anti-Epizootic Hemorrhagic Necrosis Virus (EHNV) antibody diluted 1:500 in TE. Slides were then washed three times with TE, incubated in a solution of 0.3% hydrogen peroxide and 0.1% sodium azide in TE (15 min) to inactivate endogenous peroxidase, washed three more times with TE, and incubated (1.5 h at room temperature) with 50µl of goat anti-rabbit-horseradish peroxidase conjugate antibody diluted in TE with 1% BSA. Following another three washes with TE, the slides were developed with the addition of 100µl of the chromogenic solution (0.005% 3-Amino-9-EthylCarbazole and 0.001% hydrogen peroxide in substrate buffer, 20 min at room temperature), then rinsed in running tap water, counter stained with hematoxylin (5 min), and rinsed again with tap water. Once dried, coverslips were placed on the slides using an aqueous mounting medium. Nonspecific binding and endogenous peroxidase control slides were produced by omitting the primary antibody and conjugated antibody, respectively, from the protocol described above. Only when the primary anti-EHNV antibody is applied does the secondary antibody attaches and results in a brown-red staining. Anti-EHVN antibodies are known to cross-react with FV3 (Ariel *et al.*, 2010) and are thus appropriately used to detect the presence of FV3 in tissue. (Supplemental Figure S3).

PCR for FV3 DNA. Skin and liver samples were individually transferred into tissue lysis buffer, total DNA was extracted using a spin-column DNA purification procedure (Qiagen DNeasy 96) and tested for the ranavirus major capsid protein gene with single round PCR amplification (Mao *et al.*, 1997), using the primers covering the same region of the MCP gene as the MCP1 assay recommended by the Aquatic Animal Health Code (5'–GACTTGGCCACTTATGAC-3' and 5' –GTCTCTGGAGAAGAAGAA-3') (OIE, 2012). For the fecal samples, lysis buffer was added into the sample tubes and vortexed at 55ºC four times within 1 hour, then transferred to newly labeled microfuge tubes for DNA extraction.

Parameter calculation and statistical analysis. We calculated median time to onset of clinical signs and median time to death (synonym: ST_{50}), and assessed the influence of inoculum dose and body size (SV length) on: onset of clinical signs and ST_{50} (time ratios, TRs), probability of infection and probability of death (odds ratio, OR). Infection was defined as positive PCR amplification from DNA extracted from a skin or liver sample. TRs were calculated using parametric survival models with a log-logistic distribution including only groups where clinical signs or deaths occurred. ORs were calculated using a logistic regression. Analysis was performed on STATA 13.1 (Stata statistical software, Stata Corporation LP).

Results

The $TCID_{50}$ and plaque-forming units of the FV3 stock were $10^{6.33}$/ml and $10^{7.73}$pfu/ml, respectively. The LD_{50} was calculated as $10^{2.93}$ pfu/frog (95% CI: $10^{2.42}$-$10^{3.44}$ pfu/frog) (Supplemental Figure S2).

Fifteen frogs in the groups inoculated with FV3, henceforth referred to as "fatally infected", were euthanized because of severe clinical signs (n=6) or died (n=9) from the FV3 infection (Figure 2). The remaining 19 frogs (including the four control frogs) exhibited no clinical signs, except for minimal petechiation of the hind feet (less than 10 petechiae in total, one frog in $10^{3.43}$ pfu group), and mild depression but without loss of posture or reflexes (one frog in $10^{2.43}$ pfu group), both on 14 dpi only. Clinical signs included regurgitation of food items, dazed stare or mild depression, severe depression with loss of withdrawal and/or righting reflex, and widespread petechiation on the ventral surface of the skin (Table 1). An additional observation included as a clinical sign was the presence of a small (less than 0.5 cm in greatest diameter) ovoid pearly-white bead found in the water dish of four of the fatally infected frogs but never produced by any of the survivors. It consisted of proteinaceous material (mucus) admixed with myriads of Gram (-) bacterial rods and a few rafts of cells (Figure 1d-f). Although the origin of this bead could not be unequivocally determined as its production was never observed, it was assumed to have formed in the colon of terminally diseased frogs based on its feces-like shape and its histologic similarity to material present in the lumen of necrotic segments of the distal digestive tract.

Clinical signs in fatally infected frogs began between 6-14 dpi (median 11 dpi). Mortalities began on dpi 10 and ended on dpi 14 (median time-to-death, $ST_{50,}$ 12 dpi). The interval between the onset of clinical signs and death varied from 0 (< 12 hrs based on time of the previous check) to 4.5 days (median 1 day) (Table 1). The onset of clinical signs was accelerated by 15% (95% CI 5-24%, p-value=0.001) and the ST_{50} was decreased by 16% (95% CI 6-25%, p-value=0.001) with every tenfold increase in dose, when dose was $\geq 10^{2.43}$ pfu.

The whole group of frogs involved in the experimental infection, including controls, measured an average of 35.0 mm in SV length, with a median of 35.2 mm (range 29-42, mean 95% CI 33.8-36.2) (Figure 2). The fatally infected frogs averaged 32.9 mm with a median of 33.2 mm (range 29-37, mean 95% CI 31.5-34.2). Although the frogs were not measured before the experiment, negligible growth would be expected to occur in one-year-old animals over the short period of time the trial lasted (10-22 days), and thus measurements are assumed to reflect pre-infection lengths. Even accounting for the difference in size between groups as a potential confounding variable, the size of the frog (SV length) at time of death had a significant effect on the onset of clinical signs and ST_{50} (p-values <0.001 and 0.001, respectively): every mm increase in length resulted in a 6% (95% CI 3-9%) delay in onset of signs and a 5% delay in death (95% CI 2-8%) when dose was $\geq 10^{2.43}$ pfu. The odds of dying increased 23-fold (p-value=0.017) for every tenfold increase in dose. The effect of size on the probability of death, if any, could not be statically assessed (the model would not converge), possibly due to the small sample size.

PCR for ranavirus DNA was positive in the skin and liver of all fatally infected frogs (15/15); in frogs that survived, skin and liver were consistently negative. PCR was negative in the skin and liver of all control frogs (Figure 2).

PCR for ranavirus DNA was positive in the feces of 3/15 frogs (dpi 7, 8 and 11; 3-4.5 d before death) and in the skin sheds of 4/15 frogs (dpi 3, 4, 10 and 10; strongly positive 0-1.5 d prior to death) that died from FV3 infection (Table 1). Collectively, 18 feces and 6 skin sheds collected on dpi 1-10 from several of the 15 dead frogs yielded a negative PCR signal. All feces and skin sheds collected pre-inoculation and 0-22 dpi from control frogs and inoculated frogs that did not develop severe clinical signs to warrant euthanasia were negative.

Gross lesions noted at necropsy were present only in fatally infected frogs and consisted of petechial/ecchymotic hemorrhages in skin (Figure 1a-1b), free blood in oral cavity, focal to extensive hemorrhage in the wall of stomach and/or intestine (Figure 1c), blood in fecal swab, petechiae in coelomic fat bodies or testicles and an air-filled stomach.

Splenomegaly, subjectively determined in reference to the size of the spleen of control frogs, was noted both in fatally infected frogs and those that survived, but it was slightly more marked in mortalities (average 1.9 times larger than control) (Table 1, Figure 3) than in survivors (average 1.4 times larger than control). Histologic lesions were also restricted to fatally infected frogs and involved multiple tissues (Table 2, Figures 4 and 5). Amongst the frogs that survived to the end of the trial there was some evidence of hyperplasia of hematopoietic tissue in the bone marrow and spleen, renal tubular regeneration or hyperplasia and nodular proliferation of lymphocytes in the wall of the urinary bladder and colonic submucosa. Immunohistochemical staining was performed in one frog from the non-exposed (control) group, two frogs in the group that received the highest dose ($10^{5.43}$ pfu), the sole survivor in the group that received $10^{3.43}$ pfu dose, and three frogs that survived, one from each of the lowest dose groups ($10^{2.43}$, $10^{1.43}$ and $10^{0.43}$ pfu) (Table 3). Immunohistochemical staining for ranaviral antigen (cytoplasmic, usually as fine to coarse variably abundant granules) of the fatally infected frogs that received the highest dose demonstrated the presence of ranavirus antigen in and around areas of necrosis in various tissues (Table 3). In the frogs that survived, staining was observed in scattered single-cells in the connective tissue of one or more tissues (Table 3). The morphology of the immunohistochemically stained cells found in survivors, and their location (rarely in parenchymal organs like kidney or pancreas and commonly in the submucosa of a luminal organ or wall of a cavity), suggest tissue macrophages. In nervous tissue (brain and peripheral nerves), testes, ovary, oviduct and abdominal adipose tissue no staining was detected in any of the frogs. None of the tissues from the non-exposed (control) frog stained with IHC. Non-specific staining was negligible according to the antibody and conjugate internal controls.

Discussion

Our results indicate that the oral LD_{50} of FV3 in one-year-old wood frogs averaging 35 mm of SV length is $10^{2.93\ (2.42\text{-}3.44)}$ pfu/frog (Appendix C, page 194). Although wood frogs usually begin reproduction at two years of age (Duellman & Trueb, 1994) and our experimental subjects were only one-year-old, most were sexually mature (oogenesis or spermatogenesis evident histologically in19/20 frogs examined) and thus representative of the anatomy and immunophysiology of adult individuals. Our findings can probably also apply to post-metamorphic juveniles as immune system maturation occurs at metamorphosis or soon afterwards (Robert & Ohta, 2009). Extrapolations to other experimental or to natural infections should be made cautiously if environmental conditions are different from those reported here since habitat characteristics, particularly temperature, are known to influence the immune function of amphibians (Maniero & Carey, 1997). Although we conducted the experimental inoculation in early spring, a time when wood frogs are most likely to come in contact with infected carriers as they go to the ponds for mating (Brunner *et al.*, 2004), the temperature maintained during the experiment (average 21°C) was higher than the environmental temperature would have been in the wild. Statistical models of disease incorporating any of our results as parameters must account for temperature and humidity differences in the habitat of the population of interest.

Based on our statistical model, dose of FV3 was the most important factor in the length of the incubation period, the survival time, and the probability of dying. The odds of dying increased by 23-fold and both clinical signs and death occurred approximately 15% sooner per tenfold increase in dose. The lone survivor amongst the frogs that received a dose above the LD_{50} was the largest of its group, while the frog that died in the group given a dose below the LD_{50} was the smallest (Figure 2). This is probably a reflection of the crudeness of the estimate (both mortalities fall within the 95% CI for the LD_{50} dose) and the inherent variability in susceptibility of live animals to infection more than an indication that the size of the frog, as estimated by SV length, could exert an influence on survivability at a given viral concentration.

While there is no analytical or empirical evidence to support an influence of size on the probability of dying from a given dose of FV3, the incubation period and survival time may be lengthened slightly the larger the frog is. At a given dose, the onsets of clinical signs and death were delayed by approximately 6% and 5%, respectively, for each mm increase in size. Although the model supporting this finding is analytically strong and reflects what was observed in this trial, its predictive potential is very poor, particularly when applied to frogs outside the range of sizes included in this trial. For instance, the model predicts that a 51-mm frog would die 31 dpi if given $10^{4.43}$ pfu of FV3 but, based on infection of adult wood frogs of that size (unpublished data), this overestimates the ST_{50} by 17-18 days, incorrectly doubling it. The poor predictive ability could be due to our small sample size or the occurrence of the observations for both events (clinical onset and death) during such a short time interval. The observed effect of size on the ST_{50} and onset of clinical signs is probably an indirect reflection of the effect of dose: when a given dose is administered to two frogs of different sizes the larger frog necessarily receives a smaller dose proportionally to its body size. This association suggests the need to distinguish between an absolute dose (pfu in inoculum/frog) and a proportional dose (pfu in inoculum/body size in mm). Based on a logistic regression of our results, the proportional dose of FV3 in adult wood frogs would be calculated as $10^{0.08}$ pfu/mm; e.g. the LD_{50} for a group of wood frogs averaging 50 mm in length would be close to $10^{4.18}$ pfu/frog. Future experimental infections will be required to test this hypothesis.

While the skin and liver of all frogs that died were positive for ranavirus DNA by PCR, tissues from all surviving frogs were negative. Although this could suggest that infection simply failed to establish in the surviving frogs, the difference in sampling times must be taken into account: 10-14 dpi for mortalities (positive frogs) and 22 dpi for survivors (negative frogs). The virus could either have been cleared by the time the survivors were sampled, indicating complete recovery with no carrier state, or simply been present in tissues other than those tested. This corresponds to what occurs in *Xenopus laevis* frogs inoculated intraperitoneally with FV3 where the virus is undetectable in most tissues within 2 weeks of infection (Gantress *et al.*, 2003) and in the kidney 20 dpi (Robert *et al.*, 2007) and resembles the negative PCR results found in the liver and blood of wild adult green frogs (*Rana* [*Lithobates*] *clamitans*) during a natural outbreak of FV3 (Forzán & Wood, 2013). The immunohistochemical staining observed in cells in various tissues from surviving frogs, even in a frog inoculated with the lowest dose, indicates that the frogs were infected but managed to control the infection and thus survived. The morphology of the immunohistochemically stained cells and their location are suggestive of tissue macrophages. The identity of these cells, whether the material is viable virus or simply segments of viral particles, how long the antigen remains in the surviving host cells (if indeed infected) and what role it plays in transmission is beyond the scope of this study, but the presence of ranavirus antigen in survivors suggests that even low doses of the virus are capable of causing an infection and that recovered animals may serve as asymptomatic carriers.

As stated before, differences in experimental designs of previous studies make comparisons and extrapolations difficult. For instance, *Xenopus laevis* inoculated intraperitoneally with $10^{7.7}$ pfu of FV3 exhibited temporary signs of illness (anorexia, skin shedding, and cutaneous erythema of the legs) during the first week post-infection (Gantress *et al.*, 2003). Although the erythema recorded for *X. laevis* may correspond to the petechiation observed in wood frogs in our study, it is impossible to compare the time to onset of clinical signs as the dates provided in Gantress *et al.* (Gantress *et al.*, 2003) are imprecise. Regarding ST_{50}, mortality of Cope's gray tree frog tadpoles (*Hyla chrysoscelis*, family *Hylidae*, found in the southeastern United States (Dodd, 2013)) exposed to water containing FV3 began 12 dpi and reached 66% (Hoverman *et al.*, 2010), whereas mortality of tiger salamander larvae (*Ambystoma tigrinum*, family *Ambysomatidae*, found in southern Canada, the continental United States and northeastern Mexico (Duellman & Trueb, 1994) also exposed to water containing FV3 began just under 20 dpi and reached 91% (Brunner *et al.*, 2005). Although these results could suggest a difference in susceptibility between species, Cope's gray tree frogs were exposed to 10^3 pfu/ml (10^6 pfu/l) for 3-21 days whereas salamanders were exposed to

10^5 pfu/ml for 7 days. The differences in methodology make it impossible to determine whether time-to-death and mortality rate are due to inherent species differences or simply reflect a different infective dose. If the Reed-Muench method (Reed & Muench, 1938) is applied to the salamander data as reported by Brunner *et al.*, 2005 (table 1), the LD_{50} of FV3 equals $10^{3.05}$ pfu/ml: a dose very close to the concentration of FV3 that caused 66% mortalities in Cope's gray tree frogs. Hoverman *et al.* 2010 by using a consistent methodology, demonstrated that tadpoles of three different species (pickerel frog, *Rana* [*Lithobates*] *palustris*, Cope's gray tree frog, and eastern narrow-mouthed toad, *Gastrophryne carolinensis*) are not equally susceptible to mortality when exposed to FV3. The authors also concluded that oral inoculation of a known dose of FV3 (10^6 pfu) both increased mean mortality rate and sped time-to-death when compared to water-bath exposure. However, as the same viral concentration used in the direct oral infection was diluted in a 1 L water bath, the different outcomes may actually reflect the effect of dose, not of route of infection (Hoverman *et al.*, 2010).

The use of oral dosing, as opposed to the often used intraperitoneal injection, is a better approximation of what occurs under natural conditions (Gray *et al.*, 2009). Exposure to virus-loaded water is thought to achieve infection via contact with oral or branchial mucosa (Gray *et al.*, 2009). Therefore, oral administration may be just as relevant in replicating natural exposure to viral particles in water bodies as is immersion in a water bath, and possibly more relevant for terrestrial species like the wood frog. Oral dosing allows for administration of relatively precise doses, and can be effected easily in most post-metamorphic frogs and even tadpoles of some species (Wolf *et al.*, 1968; Hoverman *et al.*, 2010).

The positive PCR signal (ranavirus DNA) in feces and skin sheds of frogs that died from infection suggests that both are potential sources of transmission, particularly in the last few days before death.

Gross and histologic lesions present in frogs that died from FV3 infection resembled those reported in most other species infected with a *Ranavirus* sp (Gray *et al.*, 2009; Cullen & Owens, 2002; Cunningham *et al.*, 2007; Kik *et al.*, 2011) and involved primarily the hematopoietic cells (bone marrow, spleen, kidney and liver), renal glomeruli, and mucosal epithelium of the oral cavity, gastrointestinal tract and, to a lesser degree, urinary bladder. Necrosis of the colonic mucosa seemed to have been associated with an accumulation of mucus and bacterial overgrowth in the colon of some individuals that was shed as a cohesive pearly-white mass approximately 24 h prior to death. Whereas the main lesion reported in immunocompromised *Xenopus laevis* infected with FV3 was necrosis of the epithelium of renal proximal tubules (Robert *et al.*, 2005), damage to the epithelium of the renal tubules in wood frogs was observed only in some individuals and it was often mild. Although there may be a true difference in the type of tissue targeted by FV3 in each species, the published histopathological images of FV3 infection in *X. laevis* appear to represent hematopoietic rather than tubular necrosis (Robert *et al.*, 2005). In some of the frogs that survived the infection, and particularly in the one survivor of the group that received $10^{3.43}$ pfu of FV3, there appeared to have been hyperplasia of bone marrow hematopoietic tissue, regeneration of renal tubules and formation of small clusters of lymphocytes in the colonic and urinary bladder mucosa, suggesting an activation of the immune system during infection. Supporting this interpretation is the lymphocytic (CD8) response to FV3 that occurs in *X. laevis* (Morales & Robert, 2007), but the precise mechanism of this response in wood frogs require further investigation.

The development and characterization of this ranavirus-wood frog model is an important step to facilitate research of ranavirus infection in North American frogs. Oral inoculation, developed for this inoculation, was easily performed and allowed for the administration of precise doses. Our results include environmental parameters, clinical signs, median survival time, probability of death at a given dose, viral shedding in feces and skin sheds, gross and histological lesions and immunohistochemical staining results under controlled laboratory conditions. These findings provide transmission, infection and mortality estimates that could be incorporate into ranavirus disease models and facilitate the design of experiments to investigate the pathogenesis of ranavirus infection in North American frogs.

Acknowledgements

This work was partly funded by the Alexander Graham Bell Graduate Scholarship-Doctoral and the Canadian Cooperative Wildlife Health Centre (now Canadian Wildlife Health Cooperative).

The authors thank Dr Alexandra Reid, who kindly provided the isolate of FV3 used in the infection, Drs Marion Desmarchelier, Shannon Martinson and Jonathan Spears, Mr Chris MacQuaid and Maciez Zawadzki, and in particular Dr Jessica Thompson and Ms Sara Vazquez Quiroga, whose efforts were indispensable to the successful rearing of the wood frogs.

Table II - 1. First appearance of clinical signs, death, interval from first signs to death (ClS-Dth), presence of ranavirus DNA (PCR) in feces and skin sheds and gross lesions at necropsy of 15 wood frogs that died or were euthanized following oral inoculation Frog Virus 3 (FV3).

FV3 (pfu)	Frog	Clinical Signs (dpi)						Death (dpi)*	ClS-Dth♮	PCR (dpi)				Gross Lesions†						
										Feces		Skin shed								
		Rgt	Brb	MDpr	SDpr	Ptc	Onst			-	**+**	-	**+**	PH-S^a	B-Or^b	PH-GI^c	B-Fc^d	PH-V^e	AFS^f	Splx^g
$10^{5.43}$	1		10	11	10		10	11.5	1.5	4,6,7			**10**							2
	2			8	10	10	8	10	2	6				y	y	y				2
	3				12	12	12	12	0	2	**8**	4		y		y				3
	4			6,11		11.5	6	11.5	4.5					y		y	y			1
	5					11.5	11.5	11.5	0	4	**7**			y		y			y	1
$10^{4.43}$	6		10		11	11	10	11	1			10		y				y^h	y	1.5
	7					12	12	12	0	4		9		y						2
	8			13	14	14	13	14	1	1,6,7,9,10		4		y						2.5
	9	10			10		10	10	0	1,6		4	**10**						y	2
	10			10	11	11	10	11	1	1			**3≈**	y		y				2
$10^{3.43}$	11					12	12	12	0					y					y	1
	12		11,13		13	13	11	13	2	6			**4≈**	y		y			y	NR
	13			13	13	13	13	13	0					y				y^i	y	2.5
	14				14	14	14	14	0	7	**11**			y		y				2.5
$10^{2.43}$	16	11	13	13		13	11	14	3	2		5		y				y^h	y	2

Regurgitation of food item (Rgt), Bacteria-rich mucous bead in water dish (Brb), Dazed-stare or mild depression (MDpr), Severe depression with loss of withdrawal or righting reflex (SDpr), Petechiae in the ventral skin and/or limbs (Ptc), First onset of clinical signs (Onst); day post-infection (dpi) recorded at time of observation except when a frog died overnight, in which case 0.5 of a day was deducted. Interval between onset of clinical signs and time-of-death (Cls-Dth) of 0 indicates no signs detected on the previous check (<12 hrs earlier). † Petechiae/hemorrhage in skin (PH-S), Blood from oral cavity (B-Or), Petechiae/hemorrhage in wall of gastrointestinal tract (PH-GI), Blood in fecal swab (B-Fc), Petechiae/hemorrhage in parenchymatous organs (a, fat bodies; b, testicles), Air-filled stomach (AFS), Splenomegaly: proportional increase in size compared to age-matched control; not recorded (NR).

* Death was recorded at time of observation except when a frog died overnight, in which case 0.5 of a day was deducted.

♮ Interval between onset of clinical signs and time of death (Cls-Dth) of 0 indicates no signs detected on the previous check (<12 h earlier).

≈ Only weak positivity in PCR test for ranavirus DNA.

Table II - 2. Histologic lesions in a subset (9/15) of wood frogs that died or were euthanized due o oral inoculation with Frog Virus 3 (FV3).

		Skin		Digestive tract/Coelom				Bone marrow	Kidney			UrinaryBladder	Spleen	Liver
FV3 (pfu)	Frog	DH	EpdN	EpN-Or	SH	EpN-GI	H-Ad	HN	HN	GN	TD/N	EpN-Ur	HN	HN
$10^{5.43}$	1			y*		y		y	y	y	y*	y	y	y
	2			y	y	y	y	y*		y	y*	y	y	y
	3	y	y	y*			y	y	y	y	y*	y	y	y
$10^{4.43}$	6			y*	y	y	y	y	y		y*	NR	y	
	8		y	y		y		y*	y	y	y*	y*	y	y
	10	y					y	y	y	y		y	y	
$10^{3.43}$	12	y						y	y	y			y	y
	13	y		y	y	y		y	y	y		y*	y	y
$10^{2.43}$	16	y		y		y		y	y	y		y*	NR	y
Overall#	(%)	56	22	78(33*)	33	67	44	100	89	89	56(56*)	88(43*)	100	78

Dermal hemorrhage (DH), Epidermal necrosis (EpdN), Epithelial necrosis in oral mucosa and/or tongue (EpN-Or), Submucosal hemorrhage in gastrointestinal tract (SH), Epithelial necrosis in stomach and/or intestine (EpN-GI), Hemorrhage in coelomic adipose tissue (H-Ad), Hematopoietic necrosis (HN), Glomerular necrosis (GN), Renal tubular degeneration or necrosis (TD/N), Epithelial necrosis in urinary bladder (EpN-Ur), NR, not recorded tissue lost during processing; y, lesion is present; black space, lesion is absent.

* Probable viral inclusion bodies present.

Proportion of frogs with a lesion out of all those examined (n=9).

Table II - 1. Immunohistochemical staining in a subset of wood frogs, *Rana sylvatica* [*Lithobates sylvaticus*], inoculated orally with various doses of Frog Virus 3 (FV3) and one non-exposed (control) frog. The primary antibody used (anti-EHNV, Epizootic Hematopoietic Necrosis Virus) cross-reacts with FV3 (Ariel *et al.*, 2010). Staining (+) was found in areas of necrosis in frogs that died from the infection (d), or as scattered single cells in connective tissue with no lesions in frogs that survived (s) and were euthanized 22 dpi.

	FV3(pfu)					
	$10^{5.43}$ (d)	**$10^{3.43}$ (s)**	**$10^{2.43}$ (s)**	**$10^{1.43}$ (s)**	**$10^{0.43}$ (s)**	**0 (control)**
	n=2	n=1	n=1	n=1	n=1	n=1
Oral/nasal mucosae	**+**	**+**	-	**+**	-	-
Tongue	**+**	**+**	-	-	**+**	-
Epidermis/dermis	**+**	-	-	-	-	-
Bone marrow	**+**	-	-	-	-	-
Heart (epicardium)	**+**	NE	-	-	**+**	-
Lung	**+**	-	**+**	-	-	-
Kidney	**+**	-	**+**	-	-	-
Urinary bladder	**+**	-	**+**	-	-	-
Liver	**+**	-	-	-	-	-
Pancreas	**+**	NE	**+**	-	-	-
Stomach	**+**	**-**	-	-	-	-
Small intestine	**+**	**-**	-	-	-	-
Colon	**+**	**-**	-	-	-	-
Spleen	**+**	**-**	**+**	-	-	-

NE, not examined.

Table II - 4 (S1). Temperature and humidity during rearing (tadpoles and froglets) and experimental infection (one-year-old adult frogs) in each of three different rooms.

Developmental stage	Tadpoles / Froglets	Frogs	Frogs (trial)	Overall
Duration of stay/room	22 wks	24 wks	4 wks	
Temperature (°C)				
Average	21	21	21	21
Average min-max	20-22	21-22	21-22	21-22
Min-max	18-23	19-23	20-22	18-23
Humidity (%)				
Average	64	52	21	51
Average min-max	46-61	46-62	16-26	41-56
Min-max	27-84	17-74	10-59	10-84

Figure II - 1. Gross lesions due to Frog Virus 3 infection in adult wood frogs. Petechiae in the skin of ventrum and limbs (1a, 1b) and severe hemorrhage in the intestinal wall (1c). Histologic appearance of mucous bead produced by fatally infected frogs: clusters of cells (Hematoxylin-Eosin stain, 1d) surrounded by protein-rich mucus (Periodic Acid-Schiff stain, 1e) and myriads of Gram (-) bacterial rods (Gram stain, 1f). Bars = 50 µ.

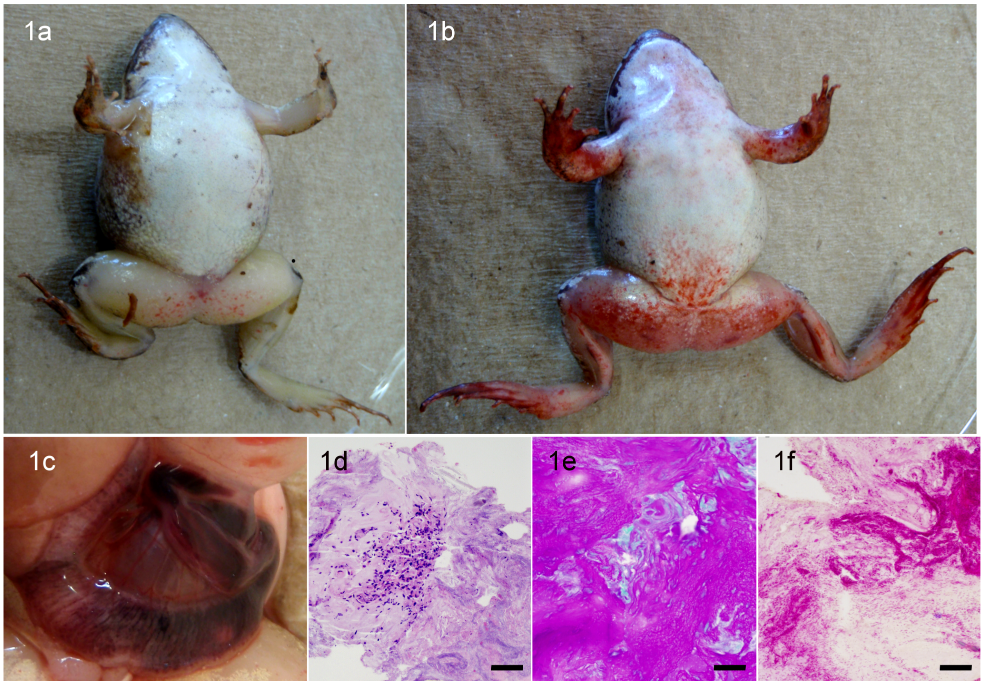
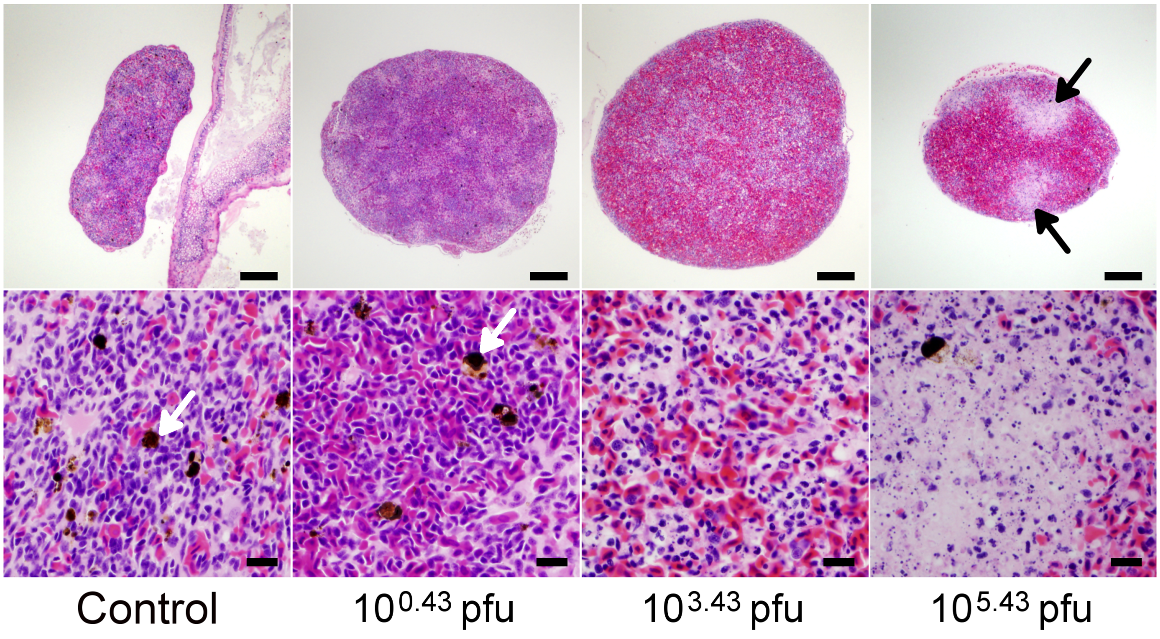


Figure II - 2. Mortalities (black) used to calculate the LD_{50} of Frog Virus 3 inoculated orally to adult wood frogs. Below each frog, from left to right: day of death post-infection, snout-vent length (SV [mm]), euthanasia due to severe disease, natural death or survival until end of trial (e, d, s, respectively), and PCR for ranavirus DNA in skin and liver (+, -). Right column: percentage of mortality, median survival time (ST_{50} [days]) and mean size (SV [mm]) of mortalities/survivors per group.

Dose (pfu/frog)																					Mortality (%)	
																					ST_{50}	SV
$10^{5.43}$																					100	
	11.5	33	d	++	10	29	d	++	12	33	e	++	11.5	31	d	++	11.5	34	d	++	11.3	32
$10^{4.43}$																					100	
	11	34	d	++	12	30	d	++	14	36	e	++	10	34	d	++	11	30	d	++	11	32.8
$10^{3.43}$																					80	
	12	32	e	++	13	36	e	++	13	37	e	++	14	34	e	++	22	39	s	--	13	34.8/39
$10^{2.43}$																					20	
	14	31	d	++	22	41	s	--	22	38	s	--	22	32	s	--	22	38	s	--	14	31/37.2
$10^{1.43}$																					0	
	22	36	s	--	22	42	s	--	22	34	s	--	22	29	s	--	22	31	s	--	n/a	34.4
$10^{0.43}$																					0	
	22	36	s	--	22	35	s	--	22	38	s	--	22	39	s	--	22	36	s	--	n/a	36.8
Control																					0	
	22	41	s	--	22	36	s	--	22	38	s	--	22	38	s	--					n/a	38

Figure II - 3. Lesions associated with oral inoculation with Frog Virus 3 in the spleen of adult wood frogs shown at subgross (upper row, 40x) and histologic (bottom row, 400x) magnifications. Spleens from control and lowest dose frogs (survivors) have no lesions and prominent melanomacrophages (white arrows). Hemorrhage, multifocal necrosis (black arrows) and loss of melanomacrophages increase in proportion to the viral dose received. Hematoxylin-eosin stain. Bars = 200 µ (top row), 20 µ (bottom row).



Control

$10^{0.43}$ pfu

$10^{3.43}$ pfu

$10^{5.43}$ pfu

Figure II - 4. Lesions associated with oral inoculation with Frog Virus 3 (FV3) in adult wood frogs. Dermal hemorrhage (a) and epidermal necrosis (b) in skin of limbs. Renal glomerular (d), hematopoietic (e) and tubular necrosis (g) with occasional intraepithelial intracytoplasmic inclusion bodies (h). Hematopoietic necrosis in the bone marrow of an infected frog (k) is presented adjacent to the bone marrow from a healthy frog (control, j); multinucleated cell with eosinophilic intracytoplasmic inclusions suggestive of viral inclusion bodies (m, arrow). Immunohistochemical staining of skin (c), hematopoietic tissue (f), a renal tubule (i), and bone marrow (l) demonstrates presence of FV3 in lesions. Bars = 50 µ (a-e, g), 20 µ (f, i) and 10 µ (h, j-m).

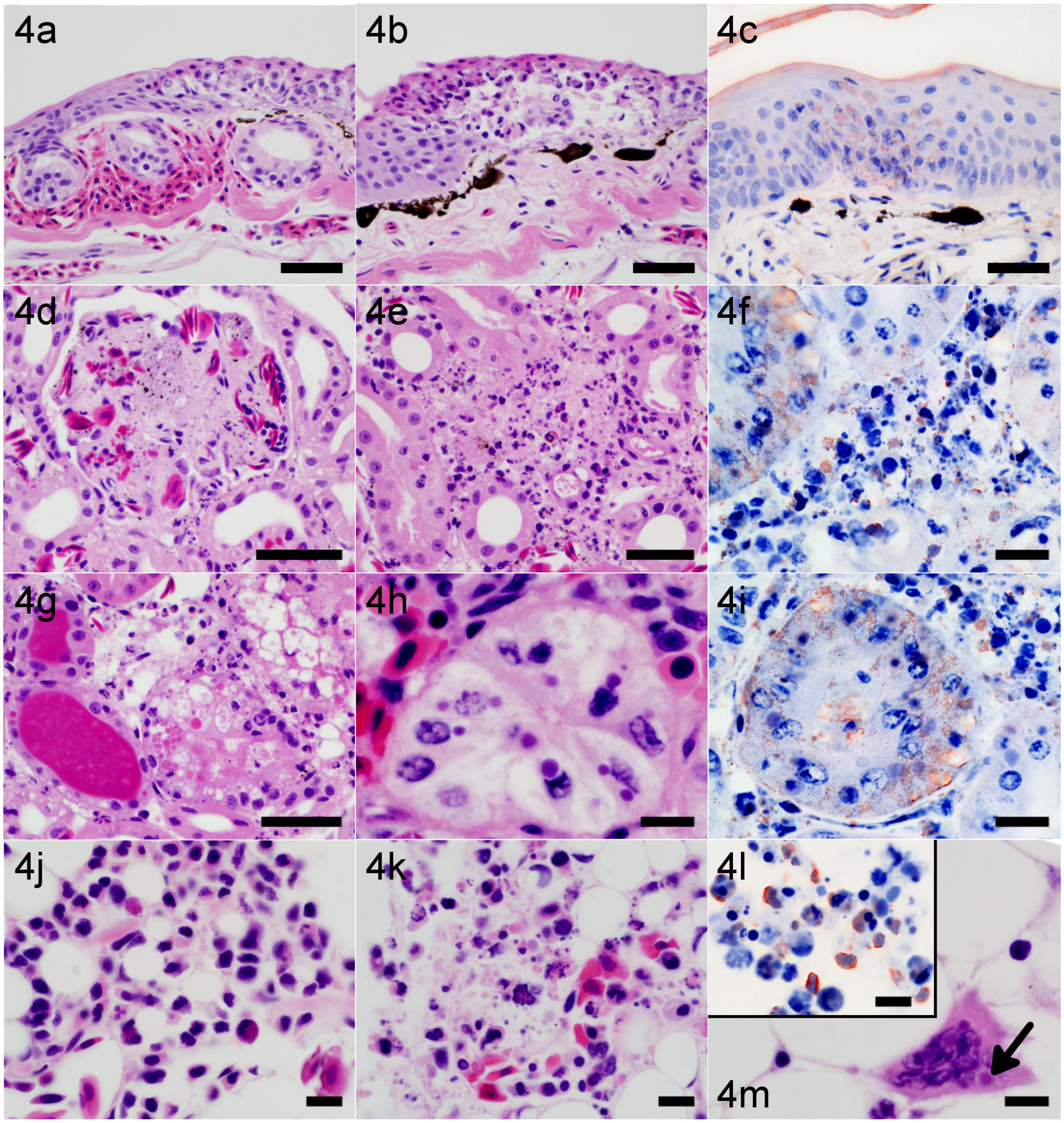


Figure II - 5. Lesions associated with oral inoculation with Frog Virus 3 (FV3) in adult wood frogs (cont.). Sections of tongue (a), stomach (c) and colon (e) from a healthy wood frog (control) presented for comparison. Severe epithelial necrosis and erosion of the tongue (b), submucosal hemorrhage in the stomach (d), and severe epithelial degeneration and necrosis with occasional probable intracytoplasmic inclusion bodies in the colon (f-g). Immunohistochemical staining of areas of mucosal epithelial degeneration and necrosis (inserts d* and g*) demonstrates the presence of FV3. Bars = 50 µ (a-f), 20 µ (g).

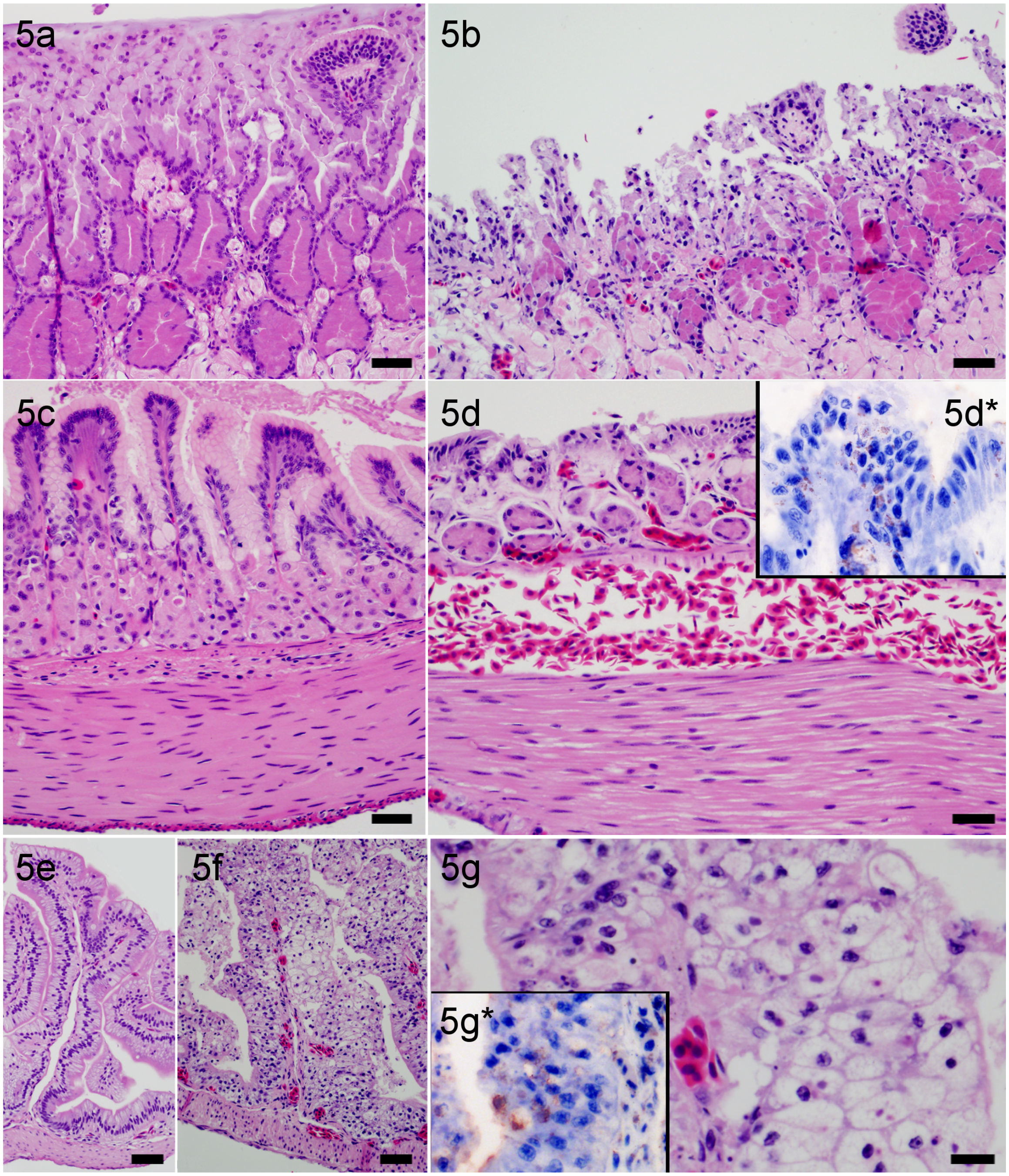


Figure II - 6 (S1). Housing of wood frogs in polycarbonate tanks and oral administration of viral inoculum. Froglets a few days after completing metamorphosis climbing the plastic plants placed on top of Petri water dishes (S1a). Adult frog sitting next to a slice of carrot in a similarly sized Petri dish (S1b) just before the experimental inoculation (1 year after hatching and 9 months after metamorphosis); bars = 20 mm. Oral administration of Frog Virus 3 diluted in minimum maintenance medium (Invitrogen) to an adult wood frog using a graded pipette after the frog's mouth has been gently opened using a smooth plastic instrument (S1c).

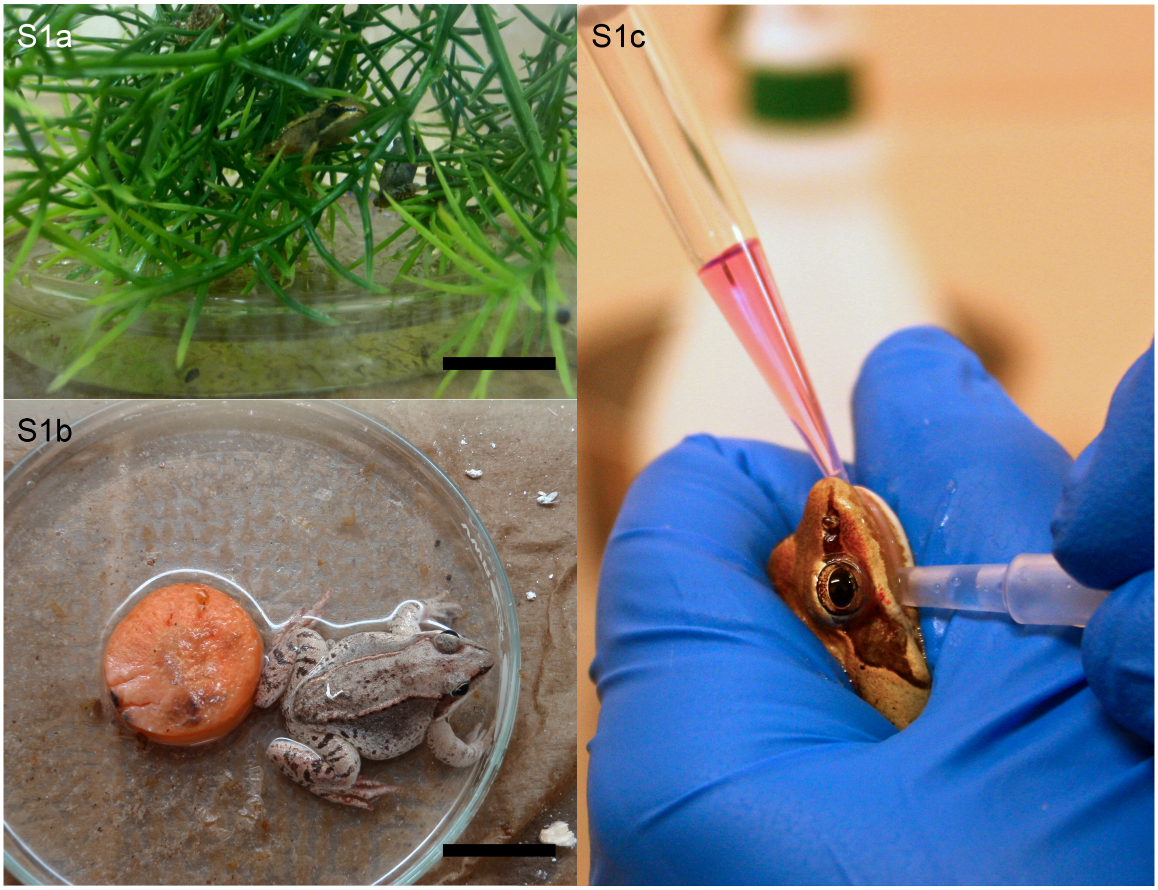


Figure II - 7 (S2). Estimated probability of death (solid line) for one-year-old adult wood frogs orally inoculated with increasing concentrations of Frog Virus 3 in plaque-forming units (pfu) as determined by a logistic regression (actual values marked by solid circle). Horizontal dashed line marks the median lethal dose (LD_{50}) threshold (50% probability of death), lighter grey dashed lines indicate the 95%CI. The LD_{50}, set as $10^{2.93}$ pfu, is demonstrated by the intercept between the horizontal dashed line and the probability of death.

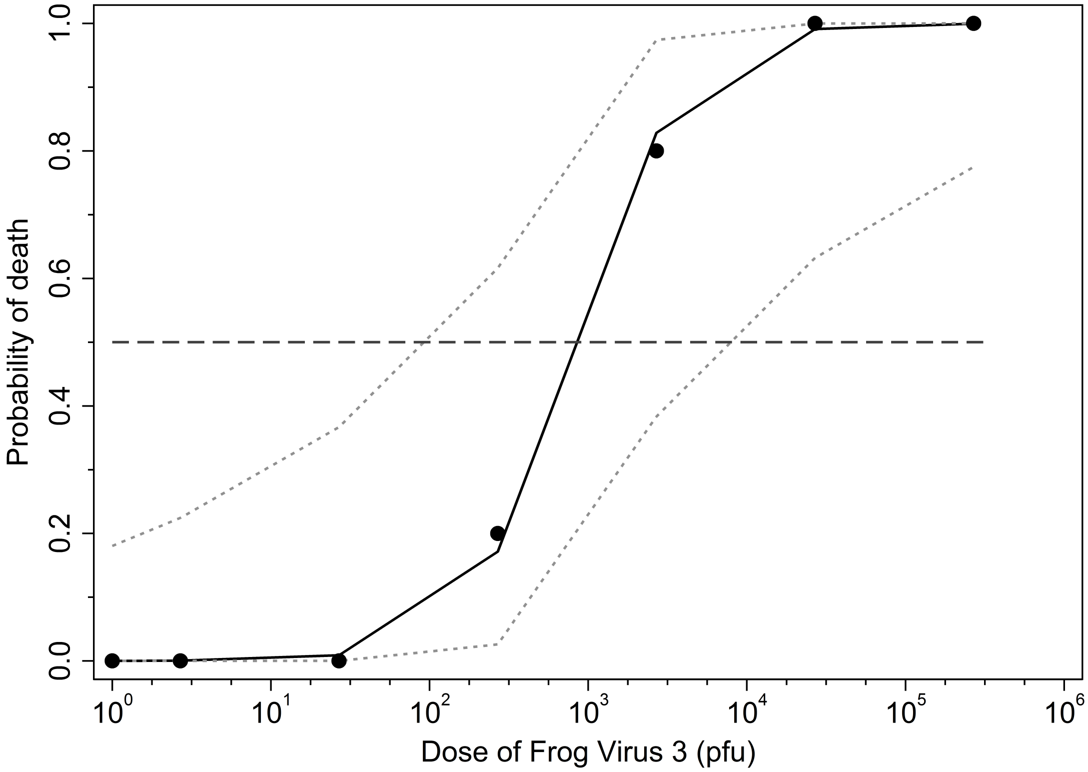
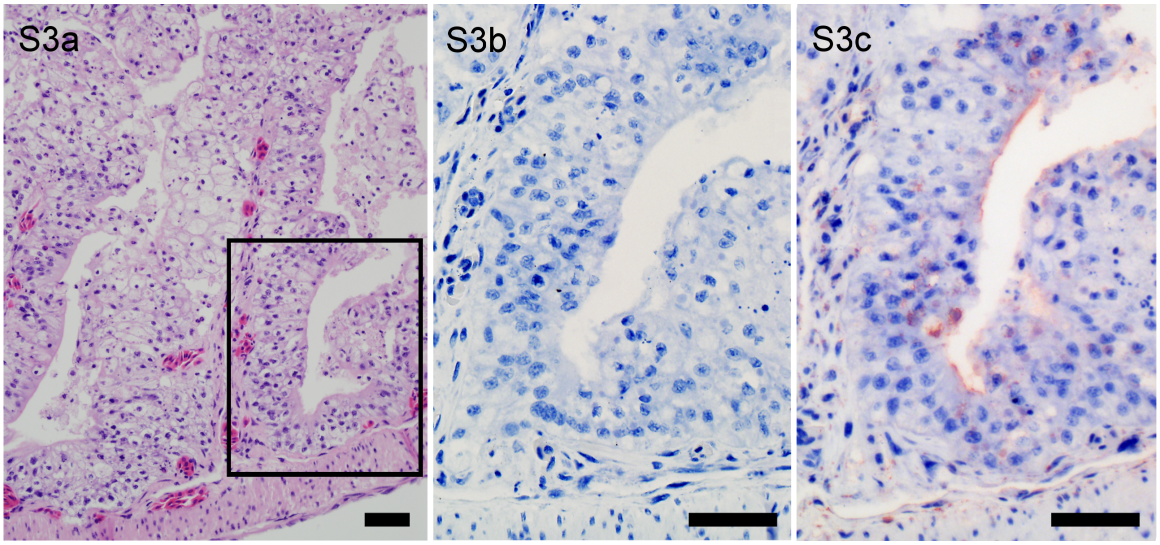


Figure II - 8 (S3). Colon of wood frog orally inoculated with Frog Virus 3. Routine staining (hematoxylin-eosin) of an area of mucosal vacuolar degeneration and early necrosis (S3a). Higher magnification of the area in the insert after immunohistochemical staining without the primary antibody (anti-EHNV, epizootic hematopoietic necrosis virus) (S3b) and with complete immunohistochemical staining that included the primary anti-EHNV antibody (S3c). Note the brown-red staining present only in immunohistochemical staining that includes the primary anti-EHNV antibody, confirming it to be specifically associated with the presence of a ranavirus in the tissue. Bars = 50 µm.



CHAPTER THREE

Clinical pathology of amphibians: a review[2]

Introduction

Clinical pathology as a tool for health evaluation can be as useful in amphibians as in higher vertebrates. The stoic nature of the amphibian patient and the great variety of species in the class make it difficult to interpret external health clues easily. Unfortunately, diagnostically relevant information for hematology and clinical chemistry of amphibians is scarce. Most reports concerning native North American species are compiled with a biologic or ecologic perspective (Burgmeier *et al.*, 2011; Coppo *et al.*, 2005; Huang CC *et al.*, 2010; Rouf MA, 1969; Solis ME *et al.*, 2007). Reports on laboratory frogs (*Xenopus* sp.) are more clinically oriented but in both instances the number of subjects studied is frequently small (Hadji- Azimi I *et al.*, 1987; Wilson S *et al.*, 2011). Published information is restricted to two orders: Anura (frogs and toads) and Caudata (salamanders and newts). Clinical pathology information is lacking for caecilians, members of the order Apoda which resemble earthworms. The study of amphibian hematology and clinical chemistry is further complicated by numerous and varied species, differences present within a species depending on its life stage, and the great influence that environmental factors have on amphibian physiology. Amphibian medicine would benefit from studies conducted under established guidelines, such as those by the American Society for Veterinary Clinical Pathology (ASVCP). The paucity of laboratory specific or even published reference intervals often necessitates the use of conspecifics for comparative assessment of parameters of interest in and individual animal. Conspecifics used as reference individuals must not only be of the same species but also of a similar age (tadpole, juvenile or adult), fed and housed in the same way, and sampled at the same time of day and under equal conditions of temperature and humidity as the individual in question. Intervals provided are for guidance only:

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[2] MJ Forzán, BS Horney. Chapter 19: Amphibians, *In*: Exotic Animal Clinical Pathology, J. Heatley and K Russell (eds), *in press.* As approved by editors, 2014.

interpretation of results must include consideration for the amphibian's clinical presentation, results from conspecifics, and the variability of differing analyzer and laboratory methodologies. Furthermore, one should be cautious of relying on reference values for a different species, even one closely related to the patient's, as this may result in misleading diagnostic information (Young S *et al.*, 2012).

Inclusion criteria for species presented here are that the data have been derived from at least 10 subjects, determined through known and acceptable methodologies and contain diagnostically relevant analytes. Information has been standardized following the recommendations of the ASVCP and is presented as Mean (Reference Interval = mean ± 2 standard deviations, or 95%CI) or Median (Reference Interval = 2.5th-97.5th quantile) for normally and non-normally distributed data, respectively. When distribution of the data was not known, normality was assumed. Data based on one to nine individuals can be found in the International Species Information System (ISIS, 2002 edition, www.isis.org) for the American toad as well as for cane toads (*Bufo* [Anaxirus] *americanus* and *B. marinus*, respectively).

Species

Modern amphibians comprise over 4000 species and can be divided in three orders, each with a distinct external morphology: Anura (frogs and toads, the archetypical amphibians), Caudata (salamanders and their aquatic counterparts, the newts, both of lacertilian shape), and Apoda (caecilians, worm-like and often blind) (Wright KM & Whitaker BR, 2001). Amphibians are becoming more common as pets, are used as models for numerous infectious and non-infectious diseases in the laboratory and have declining free-living populations. Free-living amphibian species are often studied as indicators of ecological health. Caudata and Anura, the salamander and frog groups, primarily those few native, exotic or laboratory species are most likely to be encountered by the clinician (Table 1).

Amphibian Physiology and Anatomy

Amphibians tolerate wide fluctuations in the osmolality and composition of their plasma. This adaptation is essential to conserve water since amphibian kidneys are incapable of concentrating urine above the osmolality of plasma. In general, larvae and aquatic adults, such as those in the genus *Xenopus*, excrete ammonia through the kidneys (ammonotelia), skin and gills, while terrestrial species convert ammonia in the liver into urea (ureotelia) (Duellman WE & Trueb L, 1994; Wright KM & Whitaker BR, 2001). Adults of some species of tree frogs, such as the waxy monkey tree frog (*Phyllomedusa sauvagii*), more concerned about water conservation, excrete uric acid (uricotelia) as the end product of protein metabolism even if their larvae, which are developing in a more aquatic environment, are ureotelic (Duellman WE & Trueb L, 1994; Wright KM & Whitaker BR, 2001). Endocrine organs in amphibians vary morphologically but have similar functions to those in other vertebrates. Adrenal glands (also called interrenal glands, given their location) produce three types of steroids in amphibians: aldosterone, corticosterone and cortisol (Duellman WE & Trueb L, 1994; Wright KM & Whitaker BR, 2001).

Particularly important to frogs and salamanders inhabiting the northern hemisphere are adaptations for winter survival (over-wintering or hibernation). Over-wintering frogs and salamanders have increased fibrinogen, shock proteins and glucose-transport proteins, and produce ice-nucleating proteins in the blood to guide ice formation (O'Malley B, 2005; Storey KB & Storey JM, 1986). They also accumulate low molecular weight carbohydrates (glycerol or glucose) in tissues and blood and increase plasma osmolality through dehydration. These changes lower the freezing point of tissues and ensure that ice forms in extracellular compartments, sparing cells from shearing damage (O'Malley B, 2005; Storey KB & Storey JM, 1986).

Metamorphosis occurs exclusively but not invariably in amphibians and is a complicated process that transforms an aquatic limbless larva into a tetrapod adult. During metamorphosis glucocorticoid and thyroxine levels rise, and shifts in lymphocyte populations are required to develop tolerance to a barrage of new adult antigens and a new definition of self (Duellman WE & Trueb L, 1994; Wright KM & Whitaker BR, 2001). Changes in plasma biochemistry occur in metamorphing amphibians. Serum proteins, particularly albumin, increase drastically as metamorphosis progresses in order to increase the osmotic pressure of blood and thus its water-retaining capacity (Duellman WE & Trueb L, 1994).

Individuals in captivity have relatively long life spans. Native species like the American toad (*Bufo* [*Anaxyrus*] *americanus*) that would live for 5-10 years in the wild can reach 35 years of age in captivity (Tyning TF, 1990). Exotic species that are often kept as pets, such as the bumblebee poison dart frog (*Dendrobates leucomelas*) or fire-bellied toad (*Bombina bombina*), can live for 12 years or more.

Amphibian hearts have two atria and one ventricle. The interatrial septum is fenestrated in most salamanders and complete in frogs. Blood from the caudal end of the body will pass through the kidney before entering the postcaval vein, which may affect drugs administered to the caudal aspect of the body (O'Malley B, 2005). Despite a lack of lymph nodes, there is a well-developed lymphatic vasculature that often courses parallel to large blood vessels. As in other vertebrates, lymph drains into the blood stream (Ecker A, 1889). Because of the mixing of arterial and venous blood in the single ventricle, most blood samples from amphibians, even those obtained from the heart, are considered mixed venous samples.

The site of hematopoiesis varies depending on the species. Frogs produce lymphoid, myeloid and thrombocytic cells in their bone marrow; erythrocytes are only produced in the spleen of adults while erythropoiesis occurs primarily in the liver of froglets and in the kidneys of tadpoles. Salamanders produce lymphoid and myeloid cells in their bone marrow, and erythroid cells in the spleen. In newts hematopoiesis occurs exclusively in the liver, kidney, thymus and spleen (Duellman WE & Trueb L, 1994).

Sample collection and handling

Plastic or nitrile gloves (without talcum powder), rinsed with dechlorinated water are best for handling frogs or salamanders (O'Malley B, 2005). Gloves protect the delicate skin of the patient, and reduce handler exposure to zoonotic pathogens or toxins. Amphibians can carry zoonotic pathogens such as *Salmonella* spp (CDC, 2010) and *Mycobacterium marinum* (Marthinho F & Heatley JJ, 2012), and some species produce toxic secretions (Saporito RA *et al.*, 2011). Poison frogs, found in South America (some dendrobatidis and bufonids), Madagascar (mantellids), and Australia (myobatrachids), have striking colorations that both mark them as undesirable prey and make them appealing pets (Table 1). Poison frogs have specialized skin glands that secrete toxic alkaloids. As poisonous compounds in frogs come from dietary sources, individuals maintained in captivity and fed non-poisonous arthropods will not be able to synthesize the alkaloids (Saporito RA *et al.*, 2011). The exception is the corroboree frog (*Pseudophryne* sp) which synthesizes toxic alkaloids regardless of the food source (Richards N, 2007).

Minimize handling to avoid removing the protective mucous layer that maintains skin moisture, and to decrease temperature exchange between the handler and the amphibian patient. Amphibians are poikilotherms (ectotherms) and rely on a combination of environmental temperature, heat or cold-seeking behavior, peripheral vascular control, and color changes to maintain their body temperature. An amphibian's temperature is dependent on the immediate environment and has significant influence on hematological and biochemical parameters. Thus increasing the patient's body temperature may alter test results in the sample collected (Wright KM & Whitaker BR, 2001). Collect samples from animals kept at their optimum temperature range, which depends on the species, season and life stage (Table 2).

Clinical examination and observation of the patient before sample collection should include assessing posture, blink reflex (touch the eyes gently), righting reflex (place patient on its back and watch it right itself) and withdrawal reflex (extend the limbs and watch their retraction) (Wright KM & Whitaker BR, 2001). Loss of any of these reflexes or abnormal posture may indicate serious illness and influence your decision to take a blood sample and the volume that should be collected (Heatley JJ & Johnson M, 2009). Always calculate the volume of blood that can be safely drawn from a frog or salamander to avoid causing metabolic disturbances, morbidity or mortality. Minimal total blood volumes (TBV) are 7-10% of body weight in terrestrial amphibians and 13-25% in aquatic ones (Heatley JJ & Johnson M, 2009, Figure 1). No more than 5-10% of the TBV should be drawn: usually only 5% from sick individuals and up to 10% from healthy ones (safe volume to draw, SVD).

Choice of equipment and technique of blood collection from frogs, salamanders or tadpoles depends on the size of the animal and its anatomical characteristics. Specifics for size and vein approach are given below in each section. Generally one chooses 0.3 to 3-ml syringes and 27 to 23-gauge needles. Superficial topical anesthetics such as lidocaine or benzocaine creams or gels (EMLA™ cream or Oragel®) may be used. Lithium or ammonium heparin is the preferred anticoagulant for amphibians, unless the blood is intended for bacterial culture. One may flush the needle and syringe with the liquid solution or use heparinized capillary tubes, such as Fisherbrand® Microhematocrit (Fisher Scientific Cat. no. 22-362-566) 75 mm long capillary tubes, (not to be confused with other shorter microhematocrit tubes) or green-cap Microtainer® collection tubes (BD Cat. no. 365965, coated with dry lithium heparin).

Cardiocentesis

Cardiac puncture can be used in any species, but this invasive procedure has potential life threatening complications such as cardiac standstill, atrial fibrillation and pericardial tamponade. Anesthesia is necessary and may be achieved by submerging the amphibian in a buffered solution of 0.02-0.1% (0.2-1 g/L) tricaine methanesulfonate (MS222), or a solution of benzocaine (Orajel®, or similar gels) at 0.005-0.01% for larvae and 0.02-0.03% for adults. Rinsing the patient with clean dechlorinated water after the procedure is finished will allow excretion of the MS222 and is necessary for patient recovery if benzocaine is used. Place the anesthetized tadpole, frog or salamander on its back on a moist paper towel; the cardiac pulse is either observed or detected using a doppler probe placed near the manubrium. A small 25- or 27-gauge needle is inserted into the ventricle, at a 45- to 60-degree angle to the skin, and blood is drawn either by placing a capillary tube in the hub of the needle or by gently aspirating with a 0.3-, 1- or 3-ml syringe (Heatley JJ & Johnson M, 2009; Wright KM & Whitaker BR, 2001). When successfully employed, cardiocentesis is the collection method most likely to yield the largest volume of blood. However, the amphibian heart is easily displaced within the coelomic cavity; furthermore, the bi-lobulated liver wraps tightly around the heart and may be punctured during cardiocentesis. Although the use of MS222 has been suggested to interfere with bacterial culture from samples taken under anesthesia (Cecala KK *et al.*, 2007), it is a misconception and irrelevant to routine bacterial cultures from animal samples.

Facial Vein

The facial and musculo-cutaneous veins can be sampled in ranid frogs and those with similar cranial anatomy, such as tree frogs and toads. The facial vein courses parallel to the upper jaw towards the commissure of the mouth, becoming the musculo-cutaneous vein as it passes the tympanum. Holding the frog in one hand to expose only the head, quickly insert and withdraw a small-caliber needle through the skin between the upper jawline and the rostral side of the tympanum, in the slightly sunken triangle formed by the back of the eye, the front of the tympanum and the maxillary bone (Figure 2). Insertion follows a 30° angle to the skin, in a rostro-caudal direction to access the facial vein, or in the opposite direction to access the musculo-cutaneous vein. This external collection method punctures the vein and releases blood onto the skin surface for collection with a capillary tube (Forzán MJ *et al.*, 2012). Needles should be 30-gauge for frogs weighing under 25 g, and 25- to 27-gauge for larger frogs. After collection, gentle pressure over the area achieves hemostasis. The area may be disinfected using the antiseptic spray Bactine® before or after blood collection. Depending on sampling conditions, 0.02–0.07 ml of blood (the latter loosely corresponding to the volume collected in one ammonium heparin capillary tube, Fisherbrand®) can be collected from small frogs; in large frogs,the puncture will yield larger volumes. Tilting the capillary tube earthward uses gravity to facilitate blood flow and increases the speed of collection and the blood volume obtained.   Warmer environmental temperatures facilitate blood flow. This technique does not require anesthesia and may be applied repeatedly, barring excessive sampling volumes.

Femoral and Abdominal Veins

Venipuncture of the femoral and ventral abdominal veins can be performed on relatively large amphibians. The veins should be visible through the skin unless the individual is darkly pigmented, in which case transillumination can facilitate visualization. Needles should be 27- to 30-gauge, depending on the size of the animal. While syringes can range from 0.3 to 3 ml, avoid aggressive aspiration and thus vessel collapse. The technique cannot be used in small individuals. As the ventral abdominal vein runs parallel to lymphatic vessels, lymph contamination may lead to a false interpretation of anemia, lymphocytosis or hypercalcemia (Wright KM & Whitaker BR, 2001).

Lingual Venous plexus

The lingual venous plexus may be accessed in most frogs over 25 g, but cannot be used in species with unsuitable tongue anatomy such as the pipid frogs, e.g. African clawed frogs, *Xenopus* spp.. Carefully open the mouth with a rigid but smooth instrument, such as a rubber spatula. Draw the tongue forward to expose the lingual venous plexus (Figure 3). Puncture the plexus with a 25- or 27-gauge needle and collect the blood oozing out with a capillary tube (Wright KM & Whitaker BR, 2001). Releasing the tongue and allowing the frog to close its mouth usually suffices to stop the bleeding. The contamination of the sample with saliva, a major disadvantage of this technique, can be reduced by gently rubbing the lingual surface with a cotton swab prior to sampling. This method, however, is cumbersome for the practitioner and uncomfortable for the frog.

Ventral Caudal Vein

Venipuncture of the ventral caudal tail vein, which courses immediately beneath the vertebrae, is practical for use in salamanders and newts. For salamanders weighing less than 80 g, a 27-gauge needle is needed; in salamanders over 80 g, 25-gauge needles may be used (Wright KM & Whitaker BR, 2001). Blood is slowly drawn into a 0.3- 1-ml syringe. At least two thirds of the Plethodontidae family, which includes most wild salamanders from northeastern North America, are capable of autotomy. Autotomy is the ability to willfully detach the tail at predetermined zones of breakage (cleavage points), so that if a salamander is caught by it, or if it feels threatened or injured, it may shed its tail to facilitate escape and therefore predation (Duellman WE & Trueb L, 1994). The tail may fully or completely regenerate, but coloration and internal structure may differ from the original. Therefore one must take care when using the technique on wild-caught North American salamanders as they may lose their tail during sampling. The Mexican Axolotl (*Ambystoma mexicanum*), a common pet species, does not undergo tail autotomy (Duellman WE & Trueb L, 1994).

Appendage amputation

Tail-clipping is used in salamanders and tadpoles; toe-clipping is used in frogs. These techniques are more commonly used in field studies than in a clinical setting and are the least advisable options for blood collection. The procedures are painful, so the use of local anesthetic, such as 2% lidocaine, is recommended. Application of an antiseptic, such as Bactine®, may prevent secondary bacterial infections. If toe-clipping is performed, amputation should occur at an interphalangeal joint, no more than one digit should be removed per limb, and the digit chosen must not be involved in normal behavior of the species (burrowing, climbing, amplexus, nest excavation, or propulsion) to avoid endangering the viability of the wild animal (Wright KM & Whitaker BR, 2001). Tail and toe-clipping will result in short or long-term disfiguration. Furthermore, and perhaps more importantly, toe-clipping provides either a small volume of blood or, often, none at all.

Urine collection

Collection of urine from amphibians is dependent on chance. Although most species have a small urinary bladder, catheterization or cystocentesis are not usually performed (Wright KM & Whitaker BR, 2001). Some individuals will urinate when they are captured or initially handled, but most will not, particularly if they have spent time in a transport container before being handled. Standard urinalysis tests (specific gravity/density, urea, ammonia, uric acid, etc.) can be run with amphibian samples to varying relevance. For instance, specific gravity will not indicate renal function but will reflect plasma density (see urinalysis section) (Duellman WE & Trueb L, 1994; Wright KM & Whitaker BR, 2001). Urinalysis should be performed within a few hours of collection for best results.

Sample handling

Given the small size of most amphibians, unless blood collection is terminal, only small sample volumes may be obtained. However, small amounts will suffice to obtain a complete blood cell count (CBC), make a blood smear for a differential count, evaluate morphology and detect hemoparasites, determine the red packed-cell volume (PCV), and measure total protein and other select chemical analytes. Prioritization of the sample depends on the analysis desired but the best use of a small blood sample (0.07 ml or less) is to prepare a blood smear, and determine the CBC, PCV and total protein. More voluminous samples allow for plasma biochemistry analysis. Blood can be collected directly into a capillary tube or into a syringe and then transferred to heparinized capillary tube(s) or Microtainer(s) depending upon volume.

One should prioritize analyses to derive the most diagnostic information, particularly from small samples (Figure 4). First, use a small drop from the heparinized capillary tube (Fisherbrand®) or syringe immediately after collection to make a blood smear. Actively air-dry the blood smear and fix or stain (Wright-Giemsa or Diff-Quik) within 24-48 hours for best results. Then, transfer the exact amount of blood necessary for a CBC count (generally 10-25 µl) into a purpose-specific micropipette to be mixed with pre-measured dye, such as Natt-Herrick solution (Figure 5). Finally, plug the capillary tube and centrifuge it to measure PCV and separate plasma to measure total solids (protein) with a refractometer. If more than one capillary tube is collected, pool the plasma together, skip the refractometer, and use an automated bench-top analyser for expanded biochemical analysis. Alternatively, plasma can be shipped to a diagnostic laboratory where a more accurate total protein measurement can be obtained, and additional analytes, such as urea, albumin, globulins, cholesterol, triglycerides and plasma enzymes can be measured. Diagnostic laboratories have equipment capable of processing minute amounts of plasma but need to be contacted prior to shipping to ensure the amount submitted is sufficient.

Specific analytes

Complete Blood Cell Count (CBC) (Tables 3&4):

Hemocytometry remains the most accurate method available for the amphibian complete blood count (including white blood cell, red blood cell, and thrombocyte determinations), as automated analyzers cannot differentiate between amphibian erythrocytes, leukocytes and thrombocytes. However, this method is time consuming (20-30 minutes for complete counts of the three blood cell types) and requires experience and skill, so it is avoided by all but the most resolute clinicians and researchers. Hemocytometry provides a better estimate than indirect methods from back-calculations on blood smears and is the appropriate way to establish reference intervals for a species. Natt-Herrick's solution can be stored in refrigeration for up to 2 years. The solution can be prepared following the original formulation (Natt MP & Herrick CA, 1952), purchased from a laboratory or university, or from a commercial provider in pre-measured pipetters.

Distinguishing between erythrocytes, leukocytes and thrombocytes of the amphibian patient can be challenging. Scanning the stained blood smear is helpful to gauge the shape and size of the species' blood cells before beginning the hemocytometer count. Although cell counting is best performed soon after collection, Natt-Herrick's solution contains formalin, so it maintains cellular integrity for several months (Maxham LA *et al.*, in press). Distinguishing cell types, however, becomes difficult in older samples as color differences are lost and leukocytes tend to aggregate and may thus be confused with thrombocytes. Aggregation or clumping of leukocytes will also make white blood cell counts less accurate.

Published complete blood cell counts in amphibians are rare; most rely on indirect determinations from smears rather than on hemocytometer counts and follow different sampling and analyzing techniques, so that their value to diagnostic investigation is minimal. Intentional or unwitting inclusion of thrombocytes when reporting WBC counts further limits validity of some reports. The clinician should be wary of reports that do not mention thrombocytes specifically, as these cells could have been mistakenly counted as lymphocytes. Lacking specific information regarding a particular amphibian species, interpretation of abnormalities usually follows generalities established for related species or, more frequently, other vertebrates. This extrapolation makes assumptions that are unproved in amphibians. The CBC and differential counts from a conspecific of similar age and maintained under equal environmental conditions will be helpful in the interpretation of results from a particular individual.

Red Blood Cells

Amphibian erythrocytes (red blood cells, RBC) vary in size based on the species but are ovoid and generally larger than those found in most other vertebrates (Thrall MA, 2006; Wright KM & Whitaker BR, 2001). Amphibian erythrocytes are nucleated, both in larvae and adults, except in a few species of salamanders, such as the slender salamanders of the genus *Batrachoseps*, where large proportions are anucleated (Wright KM & Whitaker BR, 2001). Erythrocytes become smaller and reduce their endoplasmic reticulum throughout metamorphosis. When using a Natt-Herrick solution, erythrocytes are commonly the largest cells, ovoid, with a pale staining cytoplasm and slightly darker nucleus; immature erythrocytes have a slightly darker cytoplasm, are smaller and they may be a bit rounder, but they usually retain their ovoid shape and are larger than leukocytes or thrombocytes (Figure 6).

Red blood cells are counted via hemacytometry or estimated via PCV. Cell morphology and proportional abnormalities in red blood cells, such as conspicuously immature erythrocytes (slightly smaller than other RBCs and with bluer cytoplasm) (Figure 7), and circulating intra or extracellular pathogens can be identified through microscopic examination of the blood smear.

Red blood cell counts vary amongst amphibian species, mainly because of differences in erythrocyte size, so values for one species are of little use as reference for another. Because of this difference in size, the PCV of two different species may be similar, while their RBC counts are different. For instance, RBCs in wood frogs (*Rana sylvatica* or *Lithobates sylvaticus*) are roughly twice as large as those from African (tropical) clawed frogs (*Xenopus tropicalis*) so that even if their PCV is similar (30%(19-41) vs 41%(27-54), respectively) their actual RBC numbers are quite different (0.4(0.3-0.6) and 1.5(1-2) x10^{12}/L, respectively) (Forzán MJ *et al.*, submitted; Maxham LA *et al.*, in press). A drop in the PCV, probably along with a reduced RBC count and an increase in the percentage of immature erythrocytes, has been found in intense infections with intraerythrocytic parasites, such as *Hepatozoon* spp., in wild green frogs, *Rana* [*Lithobates*] *clamitans* (Fielding *et al.*, manuscript in preparation) and with unidentified hemogregarines in Australian tree frogs, *Litoria caerulea* and *L. infrafrenata* (Young S *et al.*, 2012). In

American bullfrogs (*Rana* [*Lithobates*] *catesbeiana*) PCV tends to increase as the environmental temperature decreases in the winter (Weathers W, 1975).

Thrombocytes

Amphibian thrombocytes are often ovoid, occasionally round, and have a dark nucleus. Unlike lymphocytes, however, chromatin in thrombocytes is not randomly clumped but condensed at the center of the nucleus, and their cytoplasmic edges are seldom smooth (Figure 8). With Natt and Herrrick's stain, thrombocytes are ovoid or round, smaller than the erythrocytes, and with a pale blue cytoplasm lighter than that of the leukocytes (Figure 6). Amphibian thrombocytes are analogous to mammalian platelets. Thrombocytes are calculated via hemaocytometer counting; estimation using only direct examination of the blood smear is usually impeded by clumping and uneven distribution.

White Blood Cells

Leukocytes (white blood cells, WBC) in amphibians include lymphocytes, monocytes, eosinophils, basophils and neutrophils or heterophils (Figure 8) (Thrall MA, 2006). As neither heterophils nor neutrophils contain visible cytoplasmic granules in images recorded for amphibian species, this chapter will use the term neutrophil. Leukocytes are round, with less cytoplasm than erythrocytes or thrombocytes that stains dark blue to black and sometimes includes dark granules when stained with Natt & Herrick's solution (Figure 6). White blood cell numbers are calculated via hemocytometry. A stained smear is used to examine and differentiate each type of blood cell, determine the proportion of each type and, by relating that proportion to the WBC count obtained with the hemocytometer, establish the absolute numbers of each type of leukocyte. Morphology and proportional abnormalities in blood cells, such as toxic change or the presence of atypical lymphocytes and circulating intra- or extracellular pathogens can be identified through examination of the blood smear. Atypical cells, often difficult to categorize, are not uncommon in amphibian smears (Figures 7 and 9). Leukocytes in amphibians are thought to have similar inflammatory and immune functions as those in other vertebrates but published information on WBC counts is scattered and fragmented (Duellman WE & Trueb L, 1994; Wright KM & Whitaker BR, 2001). Attribution of specific significance to parameters outside reference intervals is challenging. Metamorphosis alters amphibian metabolism, and the elevated levels of glucocorticoids required may result in neutrophilia and lymphocytopenia (Duellman WE & Trueb L, 1994; Wright KM & Whitaker BR, 2001). However, the precise effects of metamorphosis on the numbers or proportions of circulating leukocytes are poorly described and understood, due to varying research methodologies amongst researchers and small numbers of animals per study. Similarly, an increase in the N:L (neutrophil:lymphocyte) ratio can be associated with increased plasma cortisol, and thus is considered an indicator of stress (Coddington EJ & Cree A, 2005). The N:L ratio is, however, non-specific and needs to be compared to an N:L ratio from conspecifics of the same age, sex and environmental conditions. A small study in American bullfrog tadpoles suffering from chytridiomycosis suggests that severely affected animals may have a higher proportion of neutrophils than those with mild lesions (Davis AK *et al.*, 2010). In reptiles, blue-grey intracytoplasmic viral inclusions have been reported in circulating leukocytes infected with Ranavirus (Allender *et al.*, 2006) while immunohistochemical staining has detected virus in circulating lymphocytes of European common frogs, *Rana temporaria* (Cunningham AA *et al.*, 2008). In wood frogs experimentally infected with Frog Virus 3 (*Ranavirus* sp) bright pink-red intracytoplasmic inclusions developed in circulating leukocytes (Forzán MJ *et al.*, submitted).

Haemoparasites

Hemoparasites are detected via direct examination of a dried stained blood smear or via PCR. Hemoparasites found on examination of amphibian blood smears include, but are not restricted to, intraerythrocytic gamonts of *Hepatozoon* sp. and various species of *Trypanosoma* spp. in the plasma (Figure 10). These parasites can be found in wild-caught individuals or captive-bred colonies with outdoor access. Frogs acquire *Hepatozoon* spp infections solely by feeding on infected *Culex* spp. mosquitos, while the mosquitos must bite infected individuals to become vectors (Boulianne B *et al.*, 2007). Interestingly, some frog trypanosomes remain in the renal vasculature during the night and circulate systemically only during the daytime: to detect infection, it is best to sample at mid-day (Southworth GC *et al.*, 1968). Infection with *Trypanosoma* spp. has not been linked to clinical disease.

Biochemical Panel

Most biochemical tests, including those for all analytes mentioned in this section, may be run on plasma as well as serum, so blood collected for hematology with a heparinized syringe or capillary tube may also be used for biochemistry panels. The normal color of amphibian plasma ranges from clear to yellow or even blue, as in the case of the Japanese giant salamander, *Andrias japonicus*, and the white-lipped tree frog, *Litoria infrafrenata* (Wright KM & Whitaker BR, 2001; Young S *et al.*, 2012). Plasma should be separated immediately after centrifugation and then kept refrigerated or frozen until processed or shipped to a diagnostic laboratory. Ideally, reference intervals should be determined from conspecifics not only of similar age and environment, but also sampled and tested by the same method, as lack of consistency in methodology diminishes the validity of reference intervals. Biochemical panels have been determined for only few amphibian species (Tables 5&6). Patchy information is available on alterations in other species or groups. Almost nothing is known of the diagnostic significance of hepatic, pancreatic, muscular or cardiac enzymes in amphibian blood.

Freeze-tolerant over-wintering amphibians such as wood frogs (*Rana sylvatica* [*Lithobates sylvaticus*]), spring peepers (*Pseudacris* [*Hyla*] *crucifer*) and Western chorus frogs (*Pseudacris triseriata*), accumulate low molecular weight carbohydrates (glucose or, less frequently, glycerol) in tissues and blood (Storey KB & Storey JM, 1986), and thus have a physiologic glucosuria if sampled soon after thawing or during frozen hibernation. In wood frogs that have recently thawed (one hour) after hibernation, blood glucose levels can be >400 mmol/L (>7,000 mg/dl) while glucose in individuals that have not undergone freezing is only around 1.5 mmol/L (27 mg/dl). In another freeze-tolerant species, the grey tree frog (*Hyla versicolor*), response is dependent on age: glucose levels of adults remain unchanged during freezing while glycerol concentrations increase from 6.8 mmol/L to 423 mmol/L; immature grey tree frogs have a mild increase in both glycerol (0.1 to 16.3 mmol/L) and glucose (1.46 to 25.9 mmol/L) (Storey KB & Storey JM, 1986).

Captive Cuban tree frogs (*Osteopilus septentrionalis*) are prone to obesity and corneal lipidosis, which are associated with marked increases in serum cholesterol and triglycerides. Cuban tree frogs affected with corneal lipidosis have cholesterol levels averaging 27.5 mmol/L or 1,062 mg/dl, while cholesterol in wild-caught, non-affected frogs averages 3.86 (0-8.26, 95%CI) mmol/L or 149 (0-319, 95%CI) mg/dl (Shilton CM *et al.*, 2001). Australian green tree frogs, also known as White's tree frogs (*Litoria caerulea)* are also prone to obesity and corneal lipidosis (Wright KM & Whitaker BR, 2001), but no information regarding the cholesterol levels of affected or non-affected individuals is available.

Renal function and/or hydration status can be evaluated through measurement of nitrogen waste-products in plasma. One must measure the appropriate form of nitrogen, remembering that the type of waste product in amphibians depends on species-specific adaptations to native environments: ammonia for aquatic adults and larvae (ammonotelic), urea for adult terrestrial amphibians (ureotelic) and uric acid for some tree frogs (uricotelic) (Duellman WE & Trueb L, 1994; Wright KM & Whitaker BR, 2001).

Electrolytes, Osmolality and Blood gases

Osmolality, the concentration of active particles in an aqueous solution, in this case serum or plasma, is usually closely associated with the hydration state and the concentration of the most diagnostically important electrolytes: sodium and potassium. Various methods of measuring osmolality and electrolytes exist, most require relatively large amounts of serum or plasma, so their determination may not be feasible except for the larger amphibians.

Amphibians are tolerant of wide fluctuations in the osmolality and composition of their plasma. This adaptation results in great variability in clinical chemistry parameters depending on environmental and physiological conditions and complicates interpretation of results. Over-wintering frogs and salamanders intentionally undergo dehydration, thus healthy animals close to or in hibernation should have increased plasma osmolality (Duellman WE & Trueb L, 1994; O'Malley B, 2005). In green (White's) tree frogs (*Litoria caerulea*), reduced plasma osmolality, and sodium, potassium, magnesium and chloride concentrations were found in six cases of severe *Batrachochytrium dendrobatidis*-infection (chytridiomycosis) (Voyles J *et al.*, 2007). Wild-caught mountain yellow-legged frogs, *Rana muscosa*, infected with *B. dendrobatidis* develop hyponatremia and hypokalemia, while acid-base balance and blood gases remain unaffected (Voyles J *et al.*, 2012).

Proteins

Blood proteins (albumin, globulins, total protein, fibrinogen and others) may be measured by refractometry, biochemical means (bromcresol green or biuret method) or protein electrophoresis. Total protein in blood includes albumin and globulins and is commonly estimated from the refractive index measured with a refractometer or calculated via spectrophotometric methods (biuret reaction) used in reference laboratories. Refractometry is a quick, dependable and inexpensive way of measuring total protein in serum or plasma. Total protein results obtained with the refractometer may, however, be artificially inflated and unreliable when other solid analytes occur in high concentrations in the plasma or serum. A refractometer reading above the reference interval, or significantly higher than the reading from a conspecific, should be confirmed through chemical protein measurement as high concentrations of bilirubin, cholesterol and tryglicerides increase the refractive index of plasma (Thrall MA, 2006). The biuret reaction, used by most reference laboratories, is the most reliable method of measuring total protein, and it is recommended over refractometry if a concentration of other solid analytes such as cholesterol and triglycerids is suspected. Although changes in osmolarity may influence the total protein measured by refractometry, the effect is seldom clinically significant.

The test used by most in-house automated analyzers to measure albumin is based on the chemical binding of albumin to bromcresol green dye; and, since binding varies depending on the animal species, it may not be valid in all amphibians. Also, it is the least reliable method in two species of Australian tree frogs (Young *et al.*, 2012). If plasma is red-tinged, suggesting hemolysis, neither biuret nor bromcresol reactions will yield valid protein or albumin measurements.

Plasma electrophoresis (EPH) calculates the relative concentrations of the various protein classes and determines the concentrations of albumin and globulins when run alongside an accurate measurement of total protein concentration. This is the most accurate, most time consuming and most expensive method for measuring proteins. It requires only very small amounts of plasma or serum (~ 5 µl) and may be the most appropriate when measuring albumin and globulins in at least some species of amphibians (Young *et al.*, 2012). If the test is run on plasma, heparin and fibrinogen bands or spikes may be present. The validity of clinical parameters such as acute phase proteins or monoclonal gammopathies as determined by EPH in amphibians is unknown.

Metamorphosis requires an increase in protein concentrations from a relatively low level in tadpoles to a concentration equal to, or slightly higher than, the baseline of adults (Duellman WE & Trueb L, 1994). Total protein in plasma of American bullfrog tadpoles (*Rana* [*Lithobates*] *catesbeiana)* increases incrementally during metamorphosis from 14.6 g/L in early larval stages to 51.6 g/L in froglets (Feldhoff RC, 1971), values that are close to the upper end of the adult intervals (43 [30-56] g/L) (Coppo JA *et al.*, 2005). Albumin levels also increase during metamorphosis of bullfrogs: from 0.6 g/L in early tadpole stages to 8.9 g/L in froglets (Feldhoff RC, 1971), a concentration close to that of adult frogs (16 [9-22] g/L) (Coppo JA *et al.*, 2005). Hibernating (over-wintering) amphibians have increased fibrinogen, shock proteins and glucose-transport proteins (O'Malley B, 2005). Differential diagnoses for non-physiologic increases in total protein include active inflammation (globulin fraction) and dehydration (albumin portion). Decreased total protein may reflect a poor diet or suggest liver, gastrointestinal or renal disease (Wright KM & Whitaker BR, 2001).

Minerals, Metals and Vitamins

Calcium metabolism in some amphibian species changes with the season and life stage: in adults calcium level in plasma increases in spring and summer, decreasing in winter, while in tadpoles calcium increases as they approach metamorphosis (Stiffler DF, 1993). In free-ranging Eastern hellbenders (*Cryptobranchus alleganiensis*) (Burgmeier NG *et al.*, 2011) (Table 6) and wild-caught African clawed frogs (*Xenopus laevis*) (Wilson S *et al.*, 2011) calcium levels in plasma and serum, respectively, are slightly higher in females than males. A similar trend is suggested for American bullfrogs (*Rana* [*Lithobates*] *catesbeiana*) (Cathers T *et al.*, 1997). Although metabolic bone disease in frogs has been reported, information on calcium concentration in amphibian plasma or on the Ca:P ratio are largely unavailable. If metabolic bone disease is suspected, whole-body radiographic examination is indicated to detect folding fractures, bone cortical thinning or decreased bone density (King JD *et al.*, 2011). Metabolic bone disease in large amphibians may also be associated with a diet that includes rats or mice, as the high levels of vitamin A contained in rodents are thought to interfere with the absorption and use of vitamin D (Wright KM & Whitaker BR, 2001).

Heavy metals (Hg, Pb, Cd, Cr and Co) in whole blood of free-ranging adult Eastern and Ozark hellbenders (*Cryptobranchus alleganiensis alleganiensis* and *C. a. bishopi*, respectively) have been reported (Huang CC *et al.*, 2010). Because none of the sites sampled included individuals from both subspecies, difference in levels between the two species are difficult to distinguish from the effect of location. However, Eastern hellbenders may have higher levels of Hg and Pb, while Co may be higher in the Ozark subspecies. Ranges incorporating all animals sampled (µg/g of whole blood) are: Hg (0.08-0.65), Pb (0.013-0.180), Co (0.07-1.41), Cr (0.13-6.87), Cd (<0.002-0.11). Mercury levels in general increase proportionally to body mass and length.

Although hypovitaminosis A is a commonly mentioned clinical concern in captive amphibians, little validation or assay comparison of whole blood or plasma vitamin A has been performed in amphibian species. The syndrome, believed to be caused by a vitamin A deficiency (Pessier AP *et al.*, 2002), is incompletely characterized and lacks studies to prove causation. Similarly, little or no information is available on plasma or blood levels of Vitamin D.

Hormones

Although amphibians have been the laboratory species of choice when studying endocrinology and reviews on amphibian endocrinology exist (Denver RJ *et al.*, 2002), most of the available information has little or no clinical application. Free-ranging female whistling frogs (*Litoria ewingi)* have a marked increase in plasma corticosterone (from <1.8 to 13.8 ng/ml) after an episode of acute stress (24 hours of captivity) (Coddington EJ & Cree A, 2005), suggesting that some responses may resemble those of other vertebrates.

Urinalysis

Amphibian kidneys cannot concentrate urine above the osmolality of plasma. Therefore, in amphibians urine specific gravity/density is not an indicator of renal function but a reflection of plasma density. A more relevant analyte to measure may be ammonia as its excretion increases during metabolic acidosis, particularly following episodes of exhaustive exercise when ammonia concentration in urine may rise to over 200% the reference level (Wright KM & Whitaker BR, 2001). Crystals may be seen in urine from tadpoles fed oxalate-rich vegetables, such as spinach or kale. Development of oxalate crystals occurs in their mesonephri and usually results in death a few days after metamorphosis (Briggs RW, 1941; Forzán *et al.*, 2015). Urinalysis reference intervals are unknown for most species. Specific gravity (1.0075 [1.0007-1.0143]) and pH (6.68 [5.26-8.1]) are only available for the American bullfrog (*Rana* [*Lithobates*] *catesbeiana*) (Coppo JA *et al.*, 2005). Renal disease is one differential for the edematous amphibian (Figure 11).

Serology and PCR useful in amphibians

PCR tests for the diagnosis of chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (Bd), and ranavirus infection require a skin swab (Bd) or a tissue sample (ranavirus). Unfortunately, the most reliable sample to diagnose ranavirus infection is a lethal sample of liver: tail-clips can be diagnostic in septicemic salamanders and tadpoles, but are not advisable in subclinical individuals; toe-clips are usually non-diagnostic in juvenile or adult frogs (Forzán MJ *et al.*, 2013). Samples are placed 70% ethanol (prepared with PCR-quality distilled water) or kept dry, depending on the instructions from the diagnostic laboratory of analysis. Serological testing for exposure to various infectious agents in amphibians is sometimes performed for research but not routinely performed in the clinical setting.

Water quality parameters for amphibians

Water quality of amphibian enclosures or surrounding environment can be measured with commercial aquarium kits (Table 7). Water quality should be assessed on a regular basis for the pet or laboratory-maintained amphibian and for amphibians suffering from dermatosepticemia, generalized edema or other illnesses (Figures 11 & 12).

Table III - 1. Amphibian species commonly kept as pets or laboratory subjects. Families known to produce toxic secretions are underlined. International Union for Conservation of Nature (IUCN) status (IUCN, 2012): least concern (LC), vulnerable (VU), near threatened (NT), endangered (EN), critically endangered (CR), data deficient (DD). Tail autotomy does not occur (*, Duellman & Trueb, 1994).

Family	Common name	Scientific name	IUCN status
Ambystomatidae	Eastern Tiger salamander	*Ambystoma tigrinum*	LC
	Mexican axolotl	*Ambystoma mexicanum**	CR
	Marbled salamander	*Ambystoma opacum*	LC
	Spotted salamander	*Ambystoma maculatum*	LC
Bombinatoridae	Oriental fire-bellied toad	*Bombina orientalis*	LC
Bufonidae	African toad	*Amietophrynus regularis*	LC
	Cane toad	*Rhinella marina*	LC
	Cururu (Rococo) toad	*Rhinella schneideri*	LC
	Gulf Coast toad	*Incilius nebulifer*	LC
Calyptocephalellidae	Helmeted water toad	*Calyptocephalella gayi*	VU
Ceratophryidae	Argentine horned (Pac-man) frog	*Ceratophrys ornata*	NT
	Brazilian horned frog	*Ceratophrys aurita*	LC
	Cranwell's horned frog	*Ceratophrys cranwelli*	LC
	Pacific horned frog	*Ceratophrys stolzmanni*	VU
Dendrobatidae	Black-legged dart frog	*Phyllobates bicolor*	NT
	Dyeing poison frog	*Dendrobates azureus*	LC
	Golden poison frog	*Phyllobates terribilis*	EN
	Golfo dulce poison-dart frog	*Phyllobates vittatus*	EN
	Green& black poison dart frog	*Dendrobates auratus*	LC
	Kõkoé poison dart frog	*Phyllobates aurotaenia*	NT
	Lovely poison frog	*Phyllobates lugubris*	LC
	Strawberry poison dart frog	*Oophaga pumilio*	LC
Eleutherodactylidae	Puerto Rican coquí	*Eleutherodactylus coqui*	LC
Hemiphractidae	Andean marsupial tree frog	*Gastrotheca riobambae*	EN
	Peru marsupial frog	*Gastrotheca peruana*	LC
Hylidae	Black-eyed (Morelet's)	*Agalychnis moreletii*	CR

	tree frog		
	Bleating tree frog	*Phyllobates bicolor*	LC
	Blue Mountains tree frog	*Litoria citropa*	LC
	Broad-palmed frog	*Litoria latopalmata*	LC
	Brown tree frog	*Litoria ewingii*	LC
	Cuban tree frog	*Osteopilus septentrionalis*	LC
	Dainty green tree frog	*Litoria gracilenta*	LC
	Eastern dwarf tree frog	*Litoria fallax*	LC
	Green and golden bell frog	*Litoria aurea*	VU
	Green (White’s) tree frog	*Litoria caerulea*	LC
	Growling (Southern bell) grass frog	*Litoria raniformis*	EN
	Leaf green tree frog	*Litoria phyllochroa*	LC
	Littlejohn's tree frog	*Litoria littlejohni*	LC
	Mimic poison frog	*Ranitomeya* [*Dendrobates*] *imitator*	LC
	Splendid tree frog	*Litoria splendid*	LC
	Mountain stream tree frog	*Litoria barringtonensis*	DD
	Peron's tree frog	*Litoria peronii*	LC
	Phantasmal poison frog	*Epipedobates tricolor*	LC
	Red-eyed tree frog (Australia)	*Litoria chloris*	LC
	Red-eyed tree frog (Central America)	*Agalychnis callidryas*	LC
	Striped rocket frog	*Litoria nasuta*	LC
	Tyler’s tree frog	*Litoria tyleri*	LC
	Waxy monkey leaf frog	*Phyllomedusa sauvagii*	LC
	Whistling tree frog	*Litoria verreauxii*	LC
	White-lipped tree frog	*Litoria infrafrenata*	LC
Hyperoliidae	Argus reed frog	*Hyperolius argus*	LC
Mantellidae	Black-eared mantella	*Mantella milotympanum*	CE
	Golden mantilla	*Mantella aurantiaca*	CR
Microhylidae	False tomatoe frog	*Dyscophus guineti*	LC
	Tomatoe frog	*Dyscophus antongilii*	NT
Myobatrachidae	Great barred frog	*Mixophyes fasciolatus*	LC
	Ornate Burrowing Frog	*Limnodynastes ornatus*	LC
	Spotted grass frog	*Limnodynastes*	LC

		tasmaniensis	
	Striped marsh frog	*Limnodynastes peronii*	LC
	Sudell's frog	*Neobatrachus sudelli*	LC
Pipidae	African clawed frog	*Xenopus laevis*	LC
	African (tropical) clawed frog	*Xenopus tropicalis*	LC
	African (Zaire) dwarf frog	*Hymenochirus boettgeri*	LC
Pyxicephalidae	African bullfrog (pixie frog)	*Pyxicephalus adspersus*	LC
Ranidae	American bullfrog	*Rana* [*Lithobates*] *catesbeiana*	LC
	Northern leopard frog	*Rana* [*Lithobates*] *pipiens*	LC
	Wood frog	*Rana sylvatica* [*Lithobates sylvaticus*]	LC
Rhacophoridae	Mossy frog	*Theloderma corticale*	DD
Rhinodermatidae	Darwin's frog	*Rhinoderma darwinii*	VU
Salamandridae	California newt	*Taricha torosa**	LC
Salamandridae	Eastern newt	*Notophthalmus viridescens*	LC
	Lorestan newt	*Neurergus kaiseri**	CR
	Oriental fire-bellied newt	*Cynops orientalis*	LC
	Taricha newts	*Taricha sp**	LC

Table III - 2. Ambient temperature gradients recommended for amphibians based on the type of species and habitat (Wright KM & Whitaker BR, 2001).

Type of species	**Habitat type**	**Temperature (°C)**	
		min	**max**
Terrestrial amphibians	Tropical lowland	24	30
	Tropical montane	18	24
	Subtropical	21	27
	Temperate	18	24
	(winter hibernation)	(10)	(16)
Aquatic amphibians (includes larvae of terrestrial species)	Tropical lowland	24	30
	Tropical montane	18	24
	Subtropical	21	27
	Temperate, stream	16	21
	Temperate, pond	18	24
	(Winter hibernation)	(9)	(15)

Table III - 1. Hematological reference intervals for select anuran species: American bullfrog (*Rana catesbeiana*, Rc, Coppo JA *et al.,* 2005), Northern leopard frog (*Rana pipiens*, Rp, Rouf MA, 1969), wood frog (*Rana sylvatica*, Rs, Forzán MJ *et al.,* submitted), African clawed frog (*Xenopus laevis*, Xl, Hadji- Azimi I *et al.,* 1987), African (tropical) clawed frog (*X. tropicalis*, Xt, Maxham LA *et al.*, in press), sapito de jardín (*Bufo fernandezae*, Bf, Cabagna-Zenklusen MC *et al.*, 2011), green (White's) tree frog (*Litoria caerulea*, Lc) and white-lipped tree frog (*Litoria infrafrenata*, Li, Young *et al.*, 2012).

Analyte (abbreviation)	**Units, SI** Conventional	**American bullfrog Ħ**	**Northern leopard frog ঌ**	**Wood frog ঌ/Ħ**	**African clawed frog Ħ**	**African (tropical) clawed frog Ħ**	**Sapito de jardín ঌ**	**Green (White's) tree frog ঌ**	**White-lipped tree frog ঌ**
Data type		**Mean (Reference Interval)[a]**						**Median (Reference Interval)[b]**	
Sample size		302	12-56	26-40	10	33-41	17	80	66
WBC	x10^9/L X10^3/µl	20.5 (11.3-29.7)	5.5 (0.7-10.3)	7.65 (2.2-13.1)	4.59 (2.92-9.16)	20.7 (11.5-36.0)[b]	4.5 (1.18-7.82)	15.9 (6.7-34.9)	21 (6.5-47.9)
Absolute neutrophils	x10^9/L X10^3/µl=			0.46 (0-0.9)		11.8 (3.7-26.7)[b]	0.79 (0.43-1.15)	3.3 (0.9-7.7)	4.2 (0.6-17.1)
Relative neutrophils	%	60.9 (36.1-85.7)	26.5 (3.7-49.3)	6.83 (0.4-13.3)	26.5 (7.5-45.5)	54.7 (26.7-82.7)	19 (0-45.8)	21.5 (7-42)	20 (5-50.3)
Absolute eosinophils	x10^9/L X10^3/µl			0.09 (0-0.25)[b]		0.2 (0-1.0)[b]	0.19 (0.11-0.27)	0.4 (0-3.1)	0 (0-2)
Relative eosinophils	%	5.8 (2.6-9)	7.3 (3.1-11.5)	1.55 (0-4.5)[b]	1.2 (0-5)	1.2(0-4.0)[b]	5 (0-13.6)	2 (0-11)	0 (0-10.6)
Absolute basophils	x10^9/L X10^3/µl			0.8 (0.1-1.5)		0.4 (0-1.1)[b]	1.34 (0.78-1.9)	0 (0-1.1)	0 (0-4)
Relative basophils	%	3.5 (1.1-5.9)	4.4 (0-10.6)	10.65 (5.9-14.8)[b]	40.5 (16.5-64.5)	2.2 (0-7.0)[b]	32 (0-76.5)	0 (0-7)	0 (0-31)
Absolute lymphocytes	x10^9/L X10^3/µl			5.76 (1.3-10.2)		7.2 (2.6-11.7)	1.71 (1.15-2.27)	10.7 (3.9-27.1)	12.2 (3.2-34.7)
Relative lymphocytes	%	26.8 (17-36.6)	53.4 (23.8-83)	76.85 (63.7-90.0)	30.1 (6.3-53.9)	37.2 (11.8-60.7)	41 (0-82.68	67.5 (40.2-88)	70 (33.4-85)
Absolute monocytes	x10^9/L X10^3/µl			0.13 (0-0.35)[b]		1.0 (0.2-2.5)[b]	0.01 (0-0.03)	1.3 (0.3-4.7)	1.1 (0.1-6.9)
Relative monocytes	%	2.9 (0.7-5.1)	11 (1.4-20.6)	1.64 (0-3.4)	1.6 (0-4)	4.7 (1.0-10.0)[b]	1 (0-2.52)	7 (2-18)	6 (1-21.3)

RBC	x10^12/L X10^6/µl	0.42 (*-1.82)	0.32 (0.16-0.48)	0.41 (0.25-0.57)	0.75 (0.53-1.09)	1.5 (1.0-2.0)	0.505 (0.24-0.76)	0.74 (0.42-1.02)	0.72 (0.4-1.12)
Packed-cell volume (PCV)	%	30.1 (19.3-40.9)		29.5 (18.6-40.5)		40.8 (27.3-54.4)		38 (23-48)	30 (19.4-48.6)
Hemoglobin	g/L g/dl	68 (38.4-97.6) 6.8 (3.84-9.76)	67.5 (27.5-107.5) 6.75 (2.75-10.75)				91.8 (35.8-147.8) 9.18 (3.58-14.78)	93 (41-126) 9.3 (4.1-12.6)	70 (33-117) 7 (3.3-11.7)
Hematocrit (Ht)	%		24.65 (5.51-43.79)				27.37 (12.01-42.73)		
Thrombocytes	x10^9/L X10^3/µl		7.3 (1.1-13.5)	8.3 (1.3-15.2)	18.52 (12-24.88)	14.6 (6.8-22.5)	4.81 (0-15.1)	27.3 (13.3-49.1)	31.9 (20-62.5)
Blood pH			7.36 (7.06-7.66)						

Reference Interval = 95%CI[a] or 2.5th-97.5th Quartile[b] Distribution of original data: normal (Rc, Xt), non-normal (Rf, Lc, Li, Xt[b]), unknown (Rp, Xl). Values below zero or incompatible with life are reported as 0.

Free living = ʒ **Captive = Ħ**

Table III - 4. Hematological reference intervals for select caudatan species: Japanese newt (*Cynops pyrrhogaster*, Cp, Pfeiffer C *et al.*, 1990), Eastern and Ozark hellbenders (*Cryptobranchus alleganiensis alleganiensis*, Caa, and *C. a. bishopi*, Cab, Huang C *et al.,* 2010).

Analyte (abbreviation)	**Units, SI Conventional**	**Japanese newt ɀ**	**Eastern hellbender ɀ**	**Ozark hellbender ɀ**
Sample size		23	37-38	42
WBC	x10^9/L X10^3/µl		3.9 (2.7-5.1)	4.6 (3.7-5.5)
Relative neutrophils[h]	%	28 (3.06-52.94)	30.3 (21.3-39.2)	35.1 (29.2-41.1)
Relative eosinophils	%	4 (0-10.72)	4.1 (1-7.3)	10.9 (8.8-13)
Relative basophils	%	57 (26.3-87.7)	6.9 (4.3-9.4)	4.3 (2.7-6)
Relative lymphocytes	%	3 (0-6.84)	54.6 (43.7-65.6)	49.3 (41.8-56.8)
Relative monocytes	%	6 (0-15.58)	1.1 (0-2.3)	0.6 (0-1.3)
RBC	x10^12/L X10^6/µl	2.28 (0-5.1)		
Hematocrit (Ht)	%	40 (21.78-58.22)	36.1 (31.7-40.6)	44.3 (41.3-47.4)
Thrombocytes	x10^9/L X10^3/µl	Not identified or counted	Clumped, not counted	Clumped, not counted

All data reported as Mean (Reference Interval = 95%CI) and calculated from a single population (Cp) or various populations weighted by sample size (Caa, Cab). Distribution of original data: normal (Caa, Cab), unknown (Cp). Values below zero are reported as 0.
Free living = ɀ Captive = Ħ

Table III - 5. Biochemical reference intervals for select anuran species: American bullfrog (*Rana catesbeiana*, Coppo JA *et al.,* 2005), Cuban tree frog (*Osteopilus septentrionalis*, Os, Shilton C *et al.*, 2001), African clawed frog (*Xenopus laevis*, Xl, Hadji- Azimi I *et al.,* 1987), African (tropical) clawed frog (*X. tropicalis*, Xt, Maxham LA *et al.*, in press), green (White's) tree frog (*Litoria caerulea*, Lc) and white-lipped tree frog (*Litoria infrafrenata*, Li, Young *et al.*, 2012).

Analyte (abbreviation)	**Units, SI** (Conventional)	**American bullfrog Ħ**	**Cuban tree frog ʓ**	**African clawed frog Ħ**	**African (tropical) clawed frog Ħ**	**Green (White's) tree frog**	**White-lipped tree frog ʓ**
Sample size		302	29	166	24	80	66
Data format		**Mean (Reference Interval)^{a}**				**Median (Reference Interval)^{b}**	
Albumin/globulin		0.54 (0.3-0.78)		0.7 (0-3.28)			
Albumin	g/L	15.8 (9.2-22.4)		10 (0-35.8)			
	g/dl	1.58 (0.92-2.24)		1 (0-3.58)			
Alkaline (ALP) phosphatase	U/L	157 (155.1-158.9)		148 (19-277)			
Alanine (ALT) aminotransferase	U/L	12.4 (10.52-14.28)		21 (0-46.8)			
Amylase	U/L			270 (0-682)			
Aspartate(AST) aminotransferase	U/L			453 (0-1587)		91 (30-362)	67 (26-370)
Bilirubin, total	umol/l			1.2 (0-6.35)			
	mg/dl			0.07 (0-0.33)			
Bilirubin, indirect	umol/l			0.8(0-5.9)			
	mg/dl			0.05 (0-0.3)			
Bilirubin, direct	umol/l			0.3 (0-5.45)			
	mg/dl			0.02 (0-0.28)			
Ca	mmol/L	2.08 (1.38-2.78)		2.2 (0.9-3.49)		2.94 (2-4.4)	2.45 (1.8-4.7)
	mg/dl	8.31 (6.89-9.73)		8.9 (3.7-14.0)			
Cl	mmol/L	108.6 (96-121.2)		82.5 (67-98)			
	mEq/L	108.6 (96-121.2)		82.5 (67-98)			
Cholesterol	mmol/L	1.6 (0.88-2.32)	3.86 (0-	6.01 (0-14)			

	mg/dl	61.8 (34.0-89.6)	8.26) 149 (0-319)	232 (0-541.2)			
Creatine (CPK) phophokinase	U/L	432 (262-602)		1658 (0-6502)		470 (75-2555)*	399 (73-3420)
Creatinine	µmol/L mg/dl	42.7 (21.14-64.26) 0.48 (0.24-0.73)		35.4 (0-262.2) 0.4 (0-2.98)			
Fe	µmol/L µg/dl	25.43 (14.8-36.0) 142.1 (82.7-201.1)					
Fibrinogen	g/L mg/dl	7.9 (5.7-10.1) 268-.7 (193.1-343.54)					
Globulin	g/L g/dl			23 (0-48.77) 2.3 (0-4.88)			
Glucose	mmol/L mg/dl	2.77 (1.43-4.11) 49.9 (25.8-74.0)		2.9 (0.07-5.73) 53 (1.46-104.54)		3.6 (1.9-6) 64.9 (34.2-108.1)	3.3 (2-6.8) 59.4 (36-122.5)
Gamma glutamyl (GGT) transferase	U/L			4 (0-29.8)			
Lactate (LDH) dehydrogenase	U/L	117 (73-161)		1809 (0-4592)			
Lipase	U/L			98 (0-201)			
P	mmol/L mg/dl	2.85 (1.69-4.01) 8.82 (5.23-12.41)		2.39 (0.84-3.94) 7.4 (2.25-12.55)		1.33 (0.72-2.64)	1.3 (0.6-2.7)
K	mmol/L mEq/L	3.62 (2.2-5.04) 3.62 (2.2-5.04)		4 (1.42-6.58)		5.9 (3.2-9.5)	3.7 (1.9-3.1)
Na	mmol/L mEq/L	118.6 (96.2-141) 118.6 (96.2-141)		123 (97.2-148-.8)		110 (101-123)	106 (99-114)
Total protein	g/L g/dl	43.4 (30.2-56.6) 4.34 (3.02-5.66)		33 (7.23-58.77)	39.1 (24.2-54.0)	62 (39-85.9)	35 (18-56.3)

				3.3 (.72-5.88)		
Triglycerides	mmol/L	0.48 (0.26-0.7)	0.4 (0-1.12)	1.3 (0-3.36)		
	mg/dl	42.5 (23-61.9)	35.4 (0-99.1)	117 (0-297.38)		
Urea	mmol/L	3 (1.76-4.24)		1.8 (0-11.1)		
Urea nitrogen (BUN)	mg/dl	8.4 (4.93-11.9)		5 (0-30.8)		
Uric acid	µmol/L	79.7 (0-422.1)		11.89 (0-165.2)	25 (4-86)	12 (0-27)**
	mg/dl	1.34 (0-7.1)		0.2 (0-2.78)		

Reference Interval = 95%CI[a] or 2.5th-97.5th Quartile[b]. Data Distribution: normal (Rc, Xt), non-normal (Lc, Li), unknown (Os, Xl). Values below zero or incompatible with life are reported as 0. **n=65

Free living = ϡ Captive = Ħ

Table III - 6. Biochemical reference intervals for select caudatan species: Eastern (*Cryptobranchus alleganiensis alleganiensis*, Caa, Burgmeier N *et al.*, 2011; Huang C *et al.*, 2010) and Ozark hellbenders (*C. a. bishop,* Cab, Huang C *et al.*, 2010).

Analyte (abbreviation)	**Units, SI** (Conventional)	**Eastern hellbender**		**Eastern hellbender**	**Ozark hellbender**
Sample size		Variable ♂	Variable ♀	37-38	42
Albumin	g/L	12.2 (10.6-13.8)	11.9 (9.5-14.3)	9.8 (7.9-11.7)	11.3 (9.3-13.2)
	g/dl	1.2 (1.1-1.4)	1.2 (0.9-1.4)	1 (0.8-1.2)	1.1 (0.9-1.3)
Aspartate (AST) aminotransferase	U/L	74.52 (0-150.72)	76.48 (0-170.72)	128.1 (84.8-171.5)	147.2 (117.1-177.4)
Ca	mmol/L	2.0 (1.72-2.28)	2.7 (1.66-3.74)	2.3 (1.9-2.7)	3 (2.7-3.3)
	mg/dl	8.0 (6.7-9.0)	10.9 (6.7-15.1)	9.3 (7.7-10.8)	12.1 (11-13.3)
Cl	mmol/L mEq/L			84.3 (81.7-86.9)	80.8 (79.0-82.7)
Creatine (CPK) phophokinase	U/L	661.7 (0-2239.1)	488.52 (0-1088.7)	3703 (641-6765)	972 (0-3100)
Globulin	g/L	26 (0-65.8)	20.3 (8.5-32.1)	21.7 (19.8-23.6)	22.3 (20.3-24.2)
	g/dl	2.6 (0-6.6)	2.0 (0.8-3.2)	2.2 (2.0-2.4)	2.2 (2.0-2.4)
Glucose	mmol/L	1.29 (0.59-1.99)	1.2 (0.6-1.8)	1.5 (0.9-2.1)	1.6 (1.1-2)
	mg/dl	23.2 (10.6-35.8)	21.6 (10.8-32.4)	26.7 (31-5.6)	29.3 (21.6-37.1)
P	mmol/L	1.66 (0.8-2.52)	1.65 (0.77-2.53)	1.6 (1.2-2)	2.2 (2-2.4)
	mg/dl	5.14 (2.48-7.8)	5.1 (2.38-7.83)	4.9 (0-9.9)	6.9 (6.3-7.5)
K	mmol/L	5.07 (2.23-7.91)	4.38 (2.02-6.74)	4.2 (3.4-5.0)	5.1 (4.5-5.6)
	mEq/L	5.07 (2.23-7.91)	4.38 (2.02-6.74)	4.2 (3.4-5.0)	5.1 (4.5-5.6)

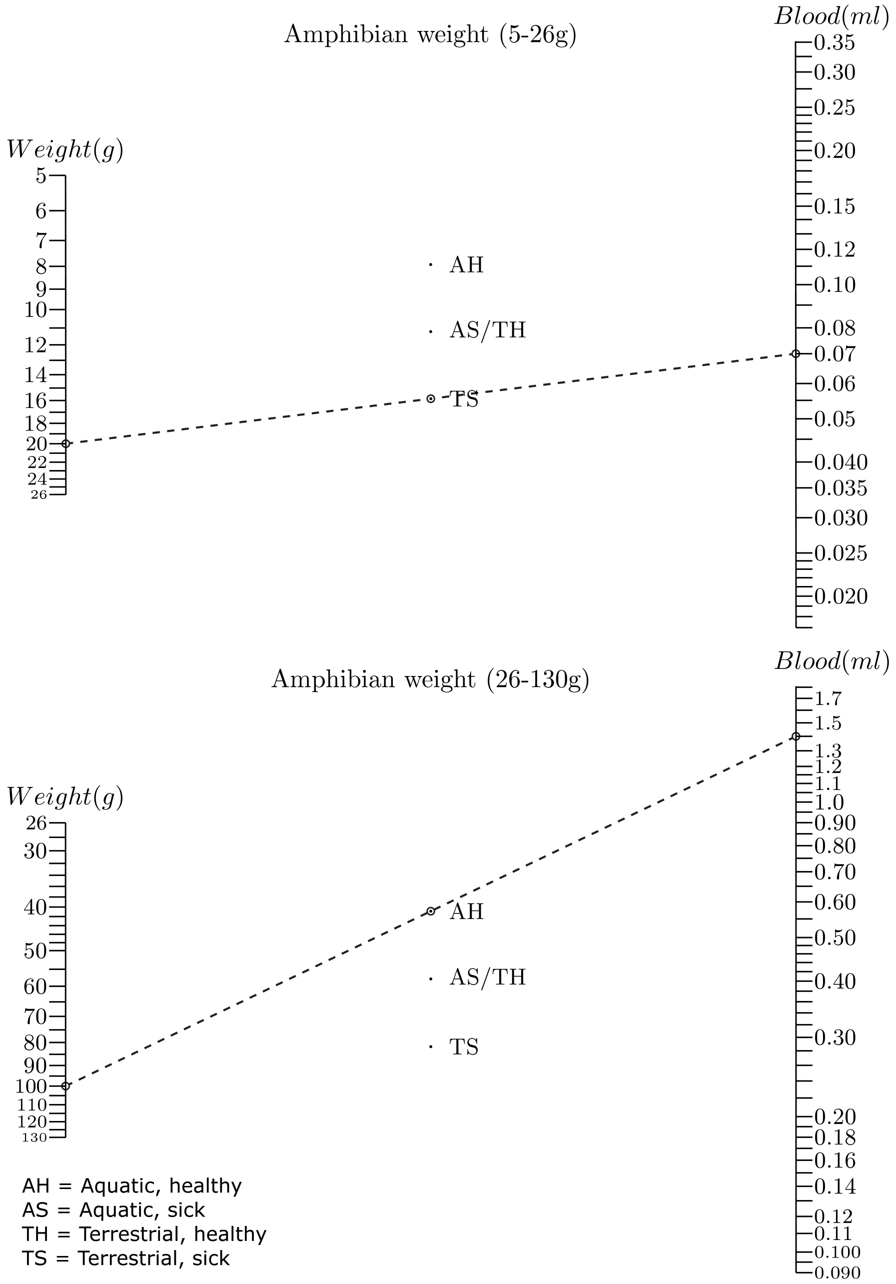
Na	mmol/L	110.83 (109.33-112.33)	111 (108.6-113.4)	106.5 (104.3-108.8)	106.3 (104.7-107.9)
	mEq/L	110.83 (109.33-112.33)	111 (108.6-113.4)	106.5 (104.3-108.8)	106.3 (104.7-107.9)
Total protein	g/L	31.5 (19.1-43.9)	31.9 (20.7-43.1)	31.8 (29-34.6)	32.5 (30.6-34.5)
	g/dl	3.15 (1.91-4.39)	3.19 (2.07-4.31)	3.2 (2.9-3.5)	3.3 (3.1-3.4)
Urea	mmol/L			0.8 (0.2-1.4)	1 (0.6-1.5)
	mg/dl			2.5 (0.7-4.2)	3 (1.8-4.2)
Uric acid	µmol/L			21.3 (13.5-29.2)	23.8 (17.3-30.3)
	mg/dl			0.4 (0.2-0.5)	0.4 (0.3-0.5)

All data reported as Mean (Reference Interval = 95%CI); calculated from various populations weighted by sample size (Caa, Cab).
Distribution of original data: normal (Caa, Cab). Values below zero are reported as 0.
Free living = ϡ Captive = Ħ

Table III - 1. Water quality parameters for amphibians in captivity (adapted from Wright & Whitaker, 2001)

Parameter	Measure
Temperature	16-20°C, Salamanders 16-24°C, temperate Frogs 22-28°C, tropical lowland Frogs 18-23°C, tropical highland Frogs
pH	6.5-8.5
Salinity	0-5 ppt
Hardness	75-150 mg/L
Alkalinity	15-50 mg/L
Dissolved oxygen	>80% saturation
Carbon dioxide	<5 mg/L
Un-ionized ammonia	<0.02 mg/L
Nitrite	<1 mg/L
Nitrate	<50 mg/L
Chlorine	undetectable

Figure III - 1. Normograph to calculate blood volume that can be drawn from an amphibian. Draw a line from the left column (body weight) through the type of species and health status (AH=aquatic healthy, AS=aquatic sick, TH=terrestrial healthy, TS=terrestrial sick): the right column indicates the volume that can be drawn safely. Conservatively, this graph is based on total blood volumes of 14% and 7% of body weight for aquatic and terrestrial species, respectively; safe volumes to draw are 5 and 10% from sick and healthy animals, respectively. Normograph constructed by R. Vanderstichel.



AH = Aquatic, healthy
AS = Aquatic, sick
TH = Terrestrial, healthy
TS = Terrestrial, sick

Figure III - 2. Facial vein venipucture. Area where 27-30-gauge needle should be quickly inserted and withdrawn is marked in pink (left). Collection with a heparinized capillary tube is aided by tilting the capillary towards the ground (centre). Hemostasis is quickly achieved after a few seconds of gentle pressure in the area (right).

Figure III - 3. Lingual plexus venipucture. Gently open the mouth of the frog with a soft instrument, such as a rubber spatula (left). Bringing the tongue forward, the venus plexi behind and beneath the tongue are exposed (right) and may be punctured with a 27-gauge needle to collect blood with a capillary tube.

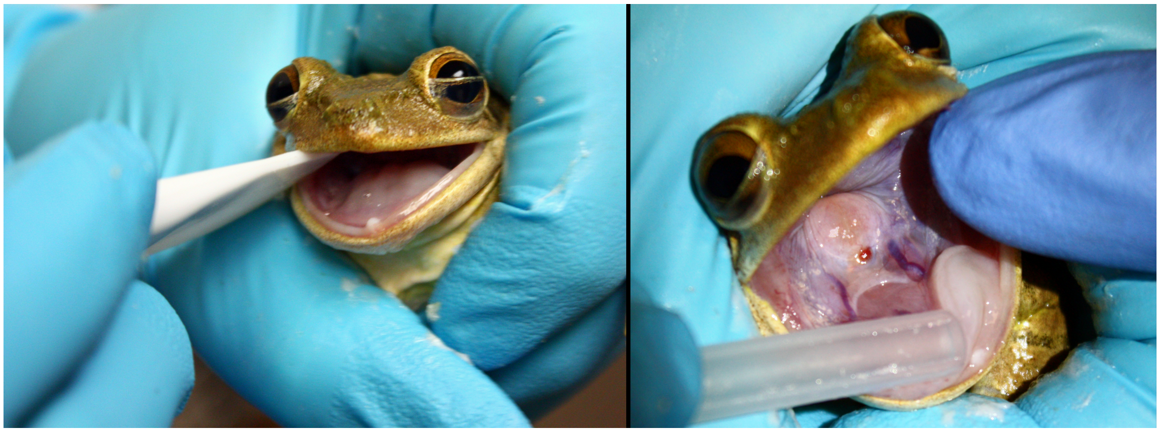


Figure III - 4. Stepwise algorithm to maximize diagnostic information obtained from amphibian blood samples.

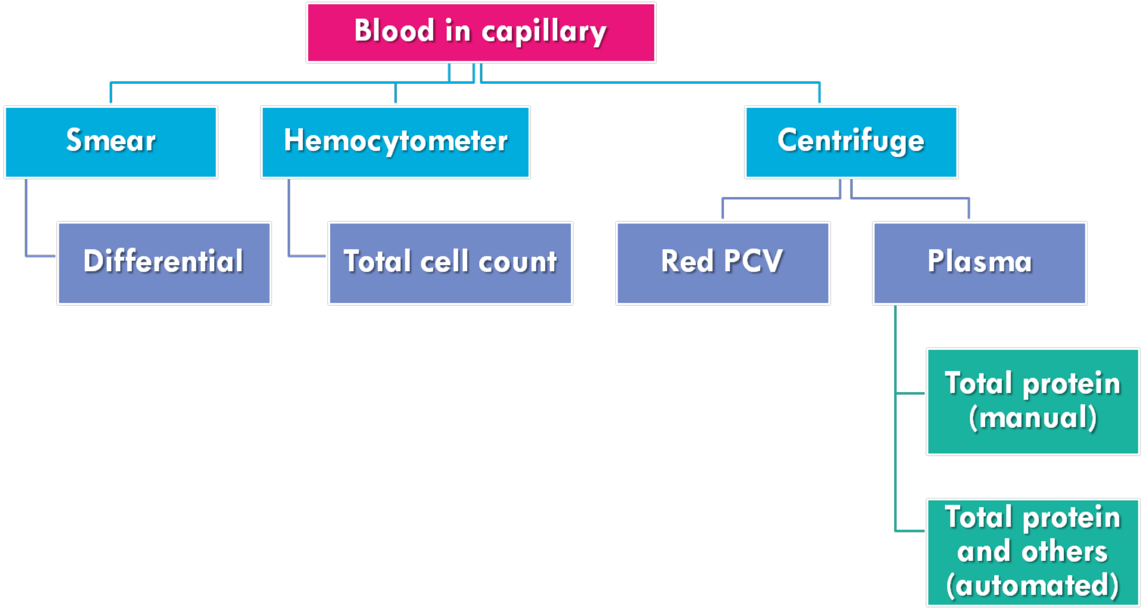


Figure III - 5. Capillary action allows for transfer of blood from syringe to heparinized capillary tube (top) and from capillary tube to Unopette® (bottom) or to any other pipette capable of measuring exactly a 20-µl volume to be used for the complete blood cell count.

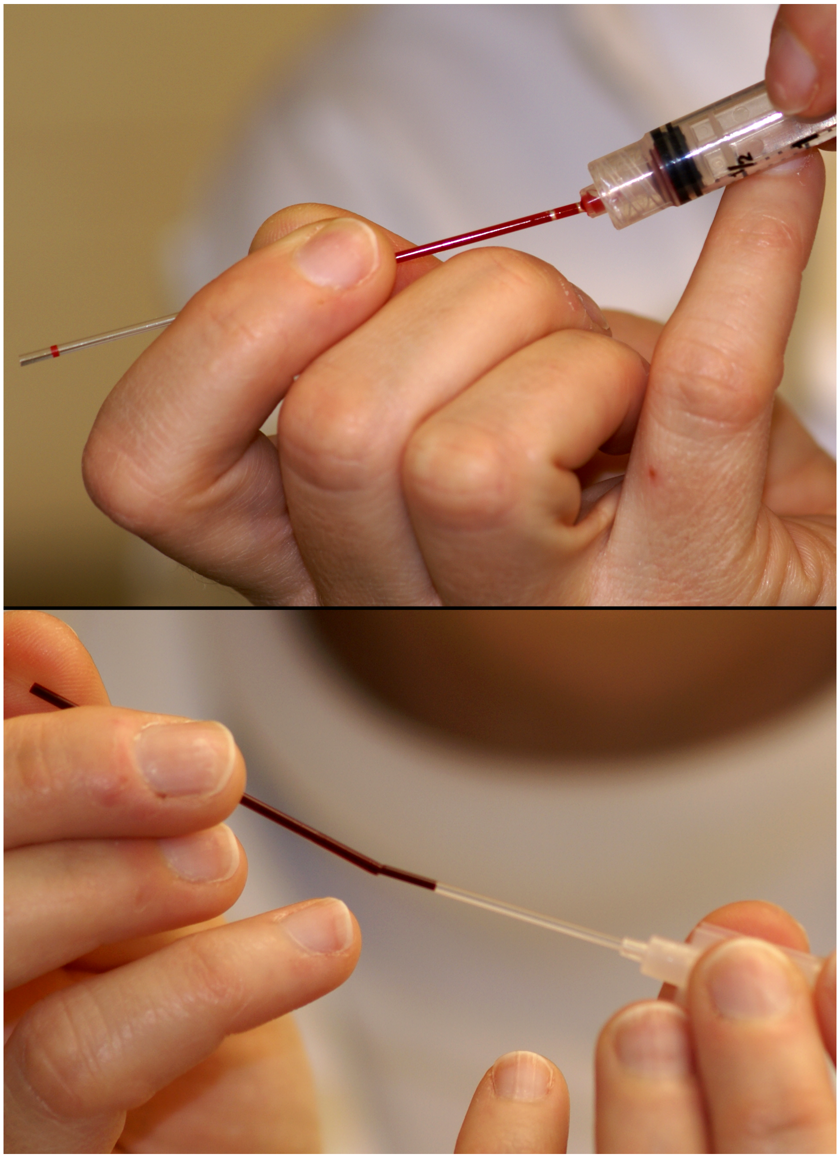


Figure III - 6. Blood cells in hemocytometer (Neubauer) chamber stained with Natt-Herrick's solution. Erythrocytes (largest, unmarked cells), leukocyte with dark granules (arrow), ovoid thrombocyte (black arrowhead) and cells that are difficult to identify (white arrowheads) that require fine adjustments of the microscope focus plane to identify: dark blue cytoplasm indicates a leukocyte (right), pale cytoplasm indicates a thrombocyte (left). Lines across the field mark the border of the chamber, 40x.

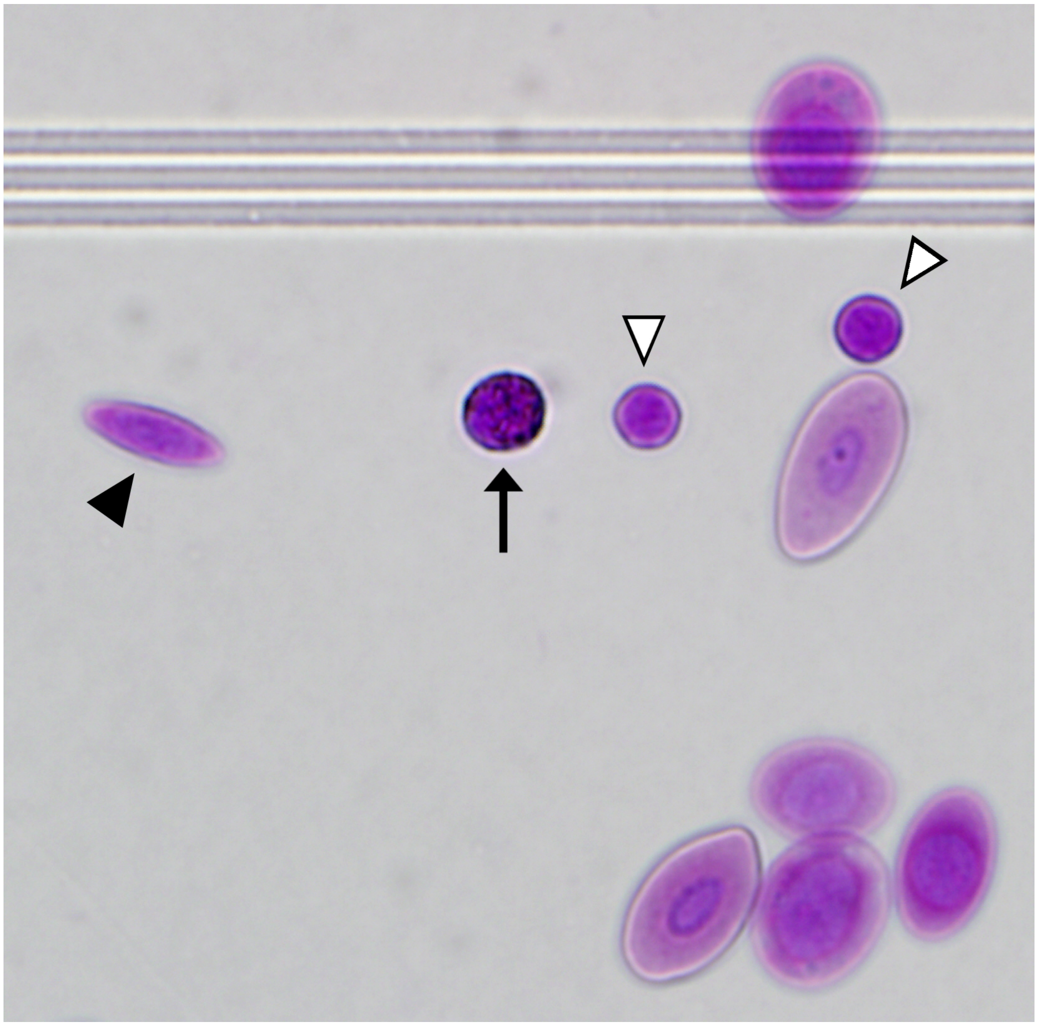


Figure III - 7. Blood smear from a green frog (*Rana* [*Lithobates*] *clamitans*). Among the three immature erythrocytes (blue-staining cytoplasm) there is an atypical cell with a large round nucleus and little deeply blue cytoplasm which may be an activate lymphocyte or an erythrocyte in an early stage in development. Wright-Giemsa stain, 100x.

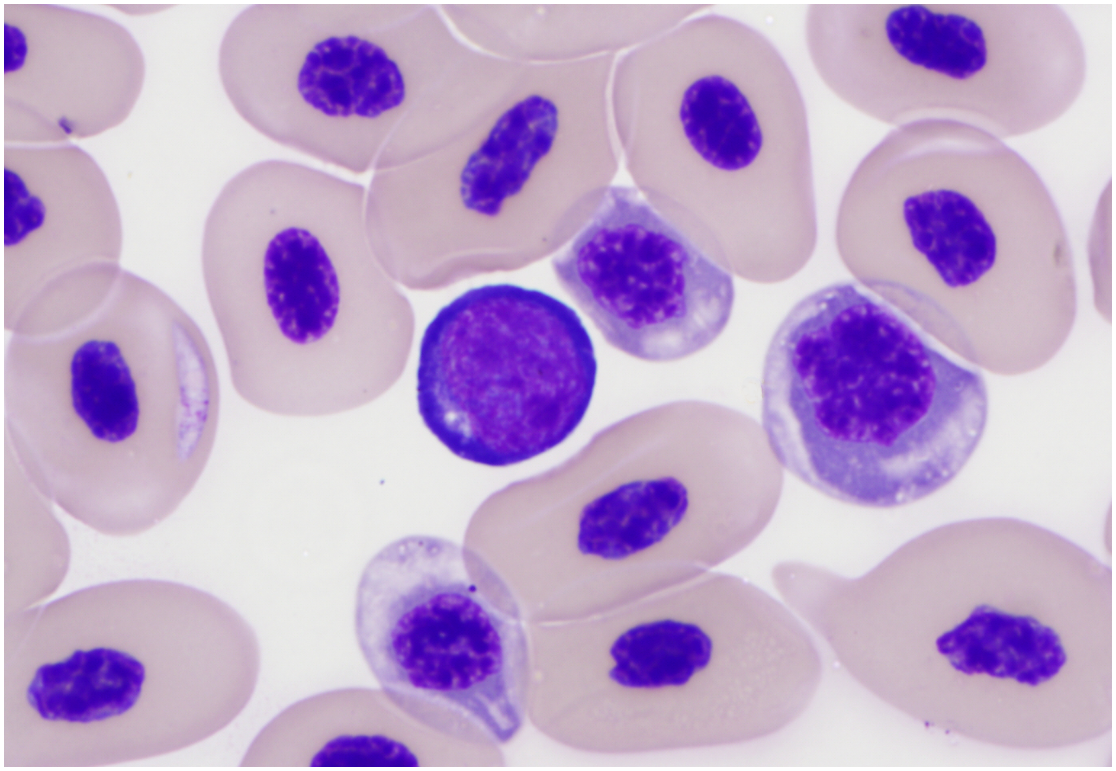


Figure III - 8. Leukocytes and thrombocytes of the green frog (*Rana* [*Lithobates*] *clamitans*). Clockwise from top left: neutrophils, eosinophil, basophil, thrombocytes (clumped), monocyte and lymphocyte. Wright-Giemsa stain, 100x.

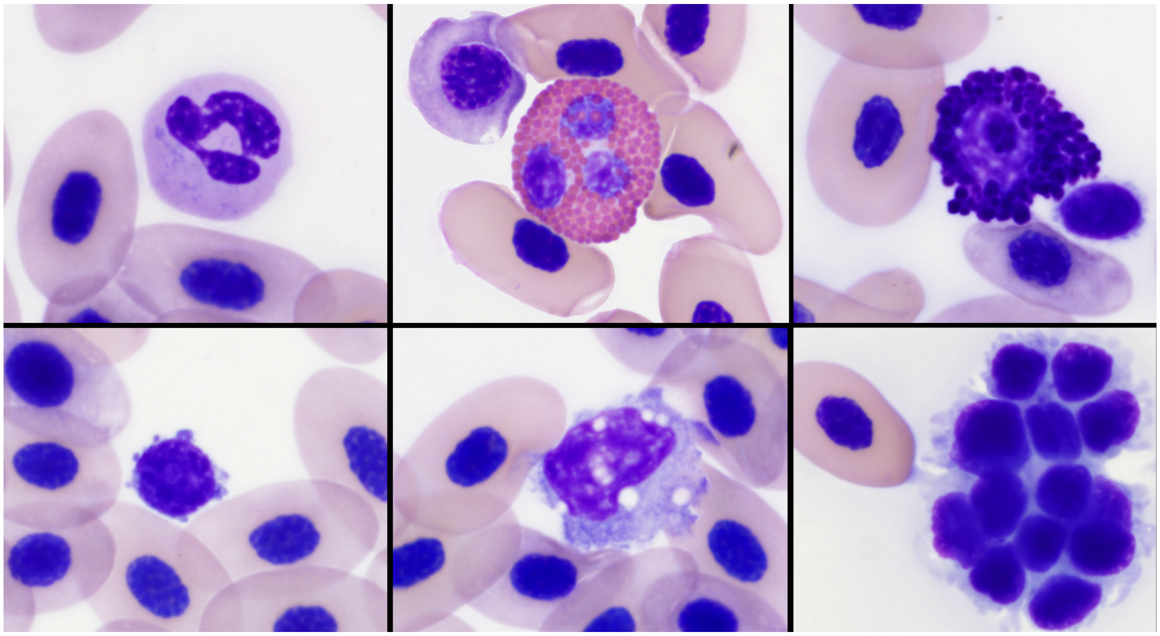


Figure III - 9. Cuban tree frog (*Osteopilus septentrionalis*) blood cell with a cytoplasm full of fine black (melanin) granules (arrow). Thrombocytes are small and ovoid, with raggedy cytoplasmic edges and often cluster together (black arrowhead); lymphocytes are round, slightly larger, with deeply blue cytoplasm and clumped nuclear chromatin. Wright-Giemsa stain, 100x.

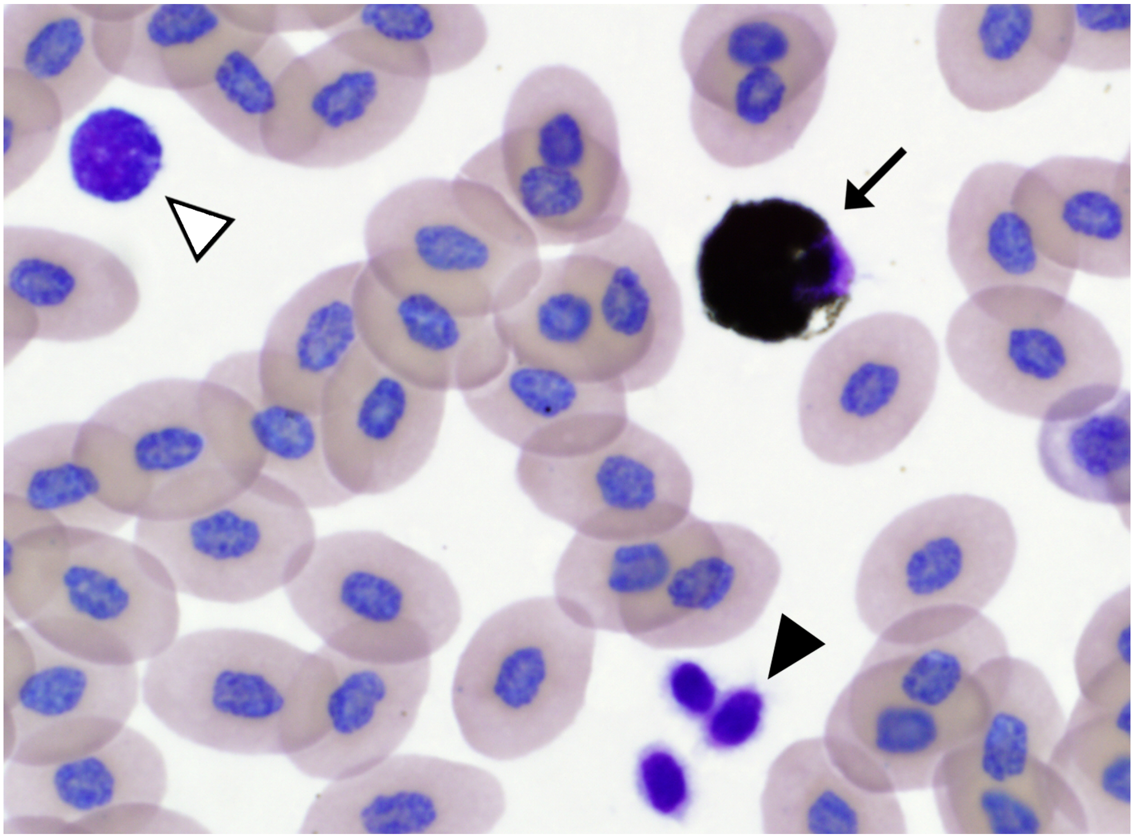


Figure III - 10. Hemoparasites of green frogs (*Rana* [*Lithobates*] *clamitans*). Gamonts of *Hepatozoon* sp in the cytoplasm of erythrocytes: intense infection accompanied by numerous immature erythrocytes with blue cytoplasm (top left); nuclear fragmentation (top middle and right) associated with *H. clamatae* infection; concurrent infection with *H. clamatae* and *H. catesbianae*. The latter does not fragment the nucleus (top right). *Trypanosoma* sp. stages in infected frogs: spherical form (bottom left, probably *Trypanosoma chattoni*) and trypomastigote stages with a visible undulating membrane (middle and right bottom, probably *T. rotatorium* and *T. pipiens*). Wright-Giemsa stain, 40x (top left) and 100x.

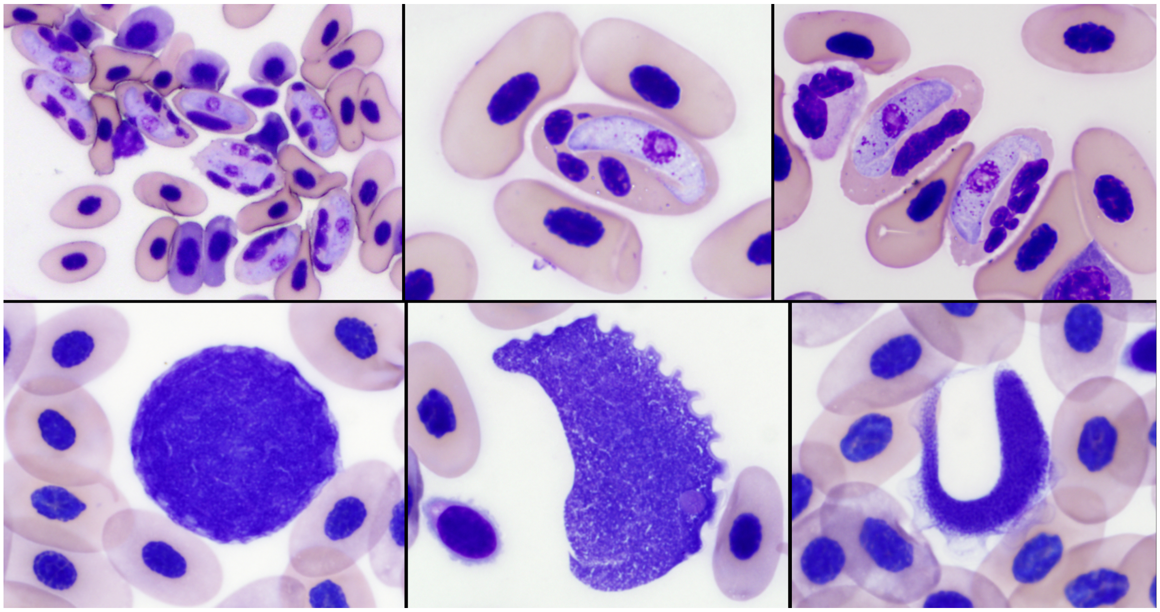


Figure III - 11. Diagnostic Diagram (tree) for amphibians with signs consistent with dermatosepticemia (erythema of the skin, petechial hemorrhages, etc.).

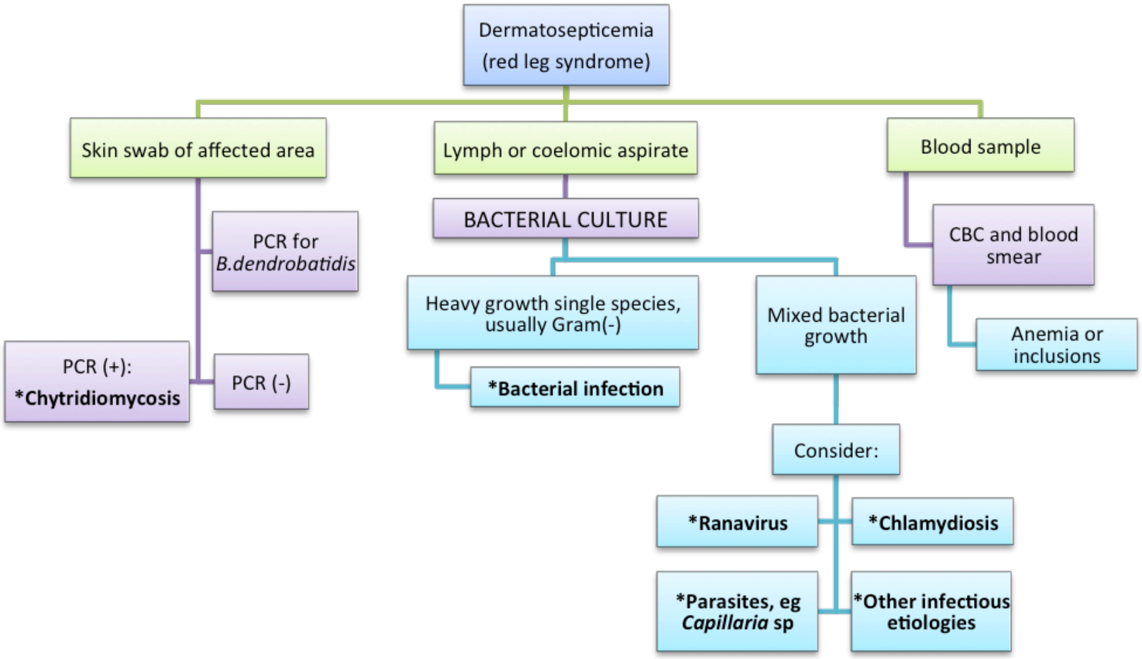
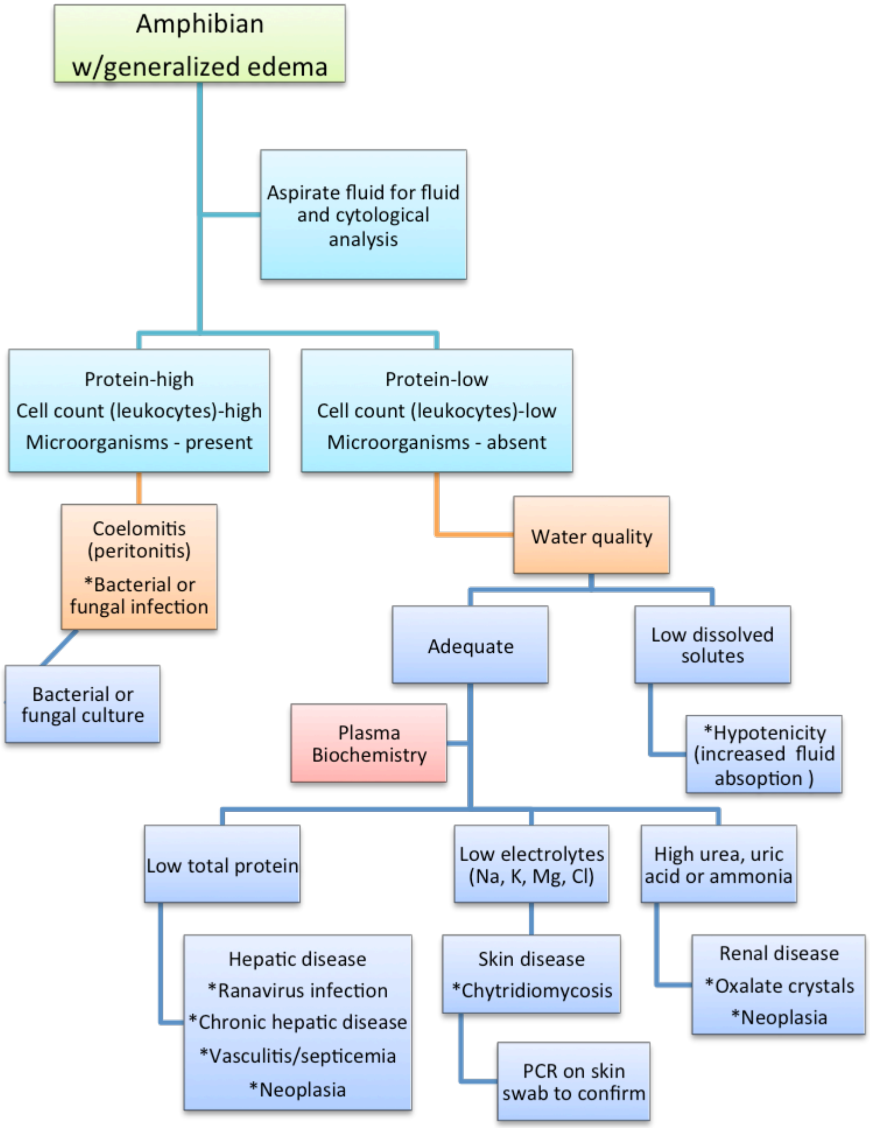


Figure III - 12. Diagnostic Diagram (tree) for amphibians with generalized edema.



CHAPTER FOUR

Hematological reference intervals for *Rana sylvatica* (*Lithobates sylvaticus*) and effect of infection with Frog Virus 3 (*Ranavirus* sp, Iridoviridae)[3]

Abstract

Background: Although the wood frog, *Rana sylvatica*, is used in research on infectious diseases of amphibians, hematological reference intervals (RIs) or response to infection have not been established.

Objectives: Determine hematological RIs for adult wood frogs and alterations associated with infection with Frog Virus 3 (FV3, *Ranavirus* sp).

Methods: Blood was collected from 40 wild-caught adult wood frogs that had been in captivity for 6 months. Complete WBC, RBC and thrombocyte cell counts (Natt-Herrick solution hemocytometry), differential WBC counts (Wright-Giemsa-stained smears), PCV and automated total cell counts (WBC+RBC+thrombocytes, Sysmex particle counting) were calculated. Concordance correlation coefficients determined agreement between hemocytometric and automated total cell counts. Thirteen frogs were orally infected with a lethal dose of 104.43 plaque-forming units of FV3 and terminally sampled 4, 9 or 14 days post-infection (dpi). Pre- and post-infection analytes for each frog were compared.

Results: Leukocyte morphology was similar to that of other amphibians and mammals. Lymphocytes were the most numerous WBC. PCV and RBC counts were similar to other frogs in the same family. Agreement was good between hemocytometry and automated total cell counts.

Infection with FV3 caused neutrophilia, increase in undifferentiated blast-like cells and relative reduction of basophils. Lymphocytes decreased at 4 and 9 dpi but increased 14 dpi. From 9 dpi onwards, nuclear deterioration and mild toxic change were present in

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[3] MJ Forzán, TG Smith, RV Vanderstichel, NS Hogan, CV Gilroy. As submitted to Veterinary Clinical Pathology, June 2015 (VCP-15-2642 – now *in press*).

neutrophils; cytoplasmic inclusion bodies were present in lymphocytes, monocytes, neutrophils and eosinophils.

Conclusion: We provide hematology RIs for *Rana sylvatica*, and report the hematological changes resulting from a lethal FV3 infection.

Key Words: blood cell counts, Frog Virus 3, *Rana sylvatica* (*LIthobates sylvaticus*), *Ranavirus* sp, reference intervals.

Introduction

Amphibians in general, and frogs and toads in particular, were proposed as appropriate indicators of environmental health almost 30 years ago (Beiswenger RE, 1988). Anuran amphibians, i.e., frogs and toads, seemed particularly useful bioindicators of the health of complicated ecosystems because of their dependence on both aquatic and terrestrial environments (Beiswenger RE, 1988). After the first wave of global reports of amphibian declines and extinctions in the early 1990s (Wake DB, 1991), the reasons for monitoring the status of amphibian populations for their own sake, although always evident, became more pressing. With an accelerated rate of extinction, and more than a third of all amphibian species threatened, some researchers believe that we are in the midst of a mass extinction - the sixth in Earth's history (Wake DB & Vredenburg VT, 2008). Behind amphibian declines are multiple causes, many associated directly or indirectly to human activities: from habitat destruction to introduction of pathogens that cause mass mortalities in naïve populations (Wake DB & Vredenburg VT, 2008). Infectious diseases, specifically *Ranavirus* sp infection and chytridiomycosis (caused by the fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans*), have had significant effects on amphibian populations (Gray MJ *et al.*, 2009; Skerratt LF *et al.*, 2007; Martel A *et al.*, 2013). Recognition of the importance of disease in declines of amphibian populations has resulted in *Ranavirus* sp infection and chytridiomycosis becoming the first, and so far only, amphibian diseases notifiable to the World Organization for Animal Health (OIE, 2008). Chytridiomycosis in particular has been identified as the cause of the rapid decline, extirpation and extinction of anuran species throughout the world (Skerratt LF *et al.*, 2007). Mass mortalities in wild and captive frogs and salamanders due to *Ranavirus* sp infection have been observed in America, Asia, Australia and Europe (Gray MJ *et al.*, 2009), and ranaviruses have been hypothetically proposed to result in extirpation if introduced to isolated frog populations (Earl JE & Gray MJ, 2015). Frog Virus 3 (FV3), the species of *Ranavirus* responsible for many of the mortality events throughout the world, has become a focus of intense research (Chinchar VG, 2002).

Given the acknowledged importance of monitoring the health of amphibian populations and the significant role infectious disease plays in population declines, it is striking how little is known of the hematologic parameters, or analytes, of even the most abundant and frequently studied frog species. Ecological studies, particularly those in the field of conservation physiology, sometimes include hematology as a tool in evaluating stress, but this is restricted to the calculation of the neutrophil to lymphocyte ratio (Davis AK *et al.*, 2008). A complete hematological profile, routinely used in human and domestic animal medicine, could prove a valuable tool in assessing amphibian health. Hematological profiles may also yield an insight into the way the immune system of infected amphibians responds to a specific infectious agent (Allender M & Fry M, 2008). But before interpreting hematological results and detecting abnormalities due to ill health, be it due to an infectious agent or any other factor, it is necessary to examine healthy individuals of the species in question to determine reference intervals (RIs) for each analyte. In order to yield useful baseline data, determination of RIs must adhere to some standardized principles. Fortunately, detailed guidelines to establish RIs in animal species have been developed by the American College of Veterinary Clinical Pathologists following and adapting methodology that is used in human medicine (Friedrichs KR *et al.*, 2012).

Hematological evaluation in amphibians is similar to that of other non-mammalian vertebrates. Because erythrocytes and thrombocytes in amphibians are nucleated, hematic counts are usually performed manually, using a hemocytometer or Neubauer chamber rather than through the automated flow cytometry techniques commonly used in humans and domestic mammals (Lassen ED & Weiser G, 2006). Hemocytometry involves the dilution of heparinized blood into a known volume of coloring solution, such as the Natt-Herrick solution originally developed in 1952 (Natt MP & Herrick CA, 1952; Campbell TW & Ellis CK, 2007). Although hemocytometry remains the gold standard in hematologic assessment of non-domestic species, it is time-consuming and requires specialized technical skills so it is infrequently included in experimental designs and veterinary clinical practice. Partial automation of hematic cell counts using flow cytometric particle counting has been proposed and successfully used in African tropical clawed frogs (*Xenopus tropicalis*) (Maxham LA *et al.*, in press) and green frogs (*Rana* [*Lithobates*] *clamitans*) (Fleming TB, 2011). Given that total flow cytometry counts are comparable to those obtained using a hemocytometer (Fleming TB, 2011; Maxham LA *et al.*, in press), and that amphibian blood, as that of other vertebrates, consists mainly of erythrocytes (>93-98%) (Fleming TB, 2011; Hadji-Azimi I *et al.*, 1987; Maxham LA *et al.*, in press; Young S *et al.*, 2012), hemocytometry can concentrate on WBC and thrombocyte counts, and the RBC count obtained by subtracting those populations from the total cell count obtained via flow cytometry (Maxham LA *et al.*, in press). Differential WBC counts can be performed on a blood smear stained with Wright-Giemsa following routine protocols since amphibian white blood cell morphology and nomenclature are very similar to those of other vertebrates (Allender M & Fry M, 2008). The wood frog, *Rana sylvatica* (*Lithobates sylvaticus*), is widely distributed in North America (Duellman WE & Trueb L, 1994), and is a member of the Ranidae, an almost cosmopolitan family of anurans. Wood frogs have become increasingly important in ecotoxicologial research both in the field (e.g. Loftin CS *et al.*, 2012) and the laboratory (e.g. Navarro-Martin L *et al.*, 2014). The involvement of wood frogs in several large mortality events due to *Ranavirus* sp (Iridoviridae) (Miller D *et al.*, 2011), and their susceptibility to infection with *B. dendrobatidis*, the fungus that causes chytridiomycosis (e.g. Longcore JR *et al.*, 2007), make them an appropriate subject to study these OIE-reportable amphibian diseases. Wood frogs were in fact proposed by participants at the First International Symposium on Ranaviruses (Minneapolis, MN, 2011) as one of two species of Ranidae on which research should be focused (Lesbarrères D *et al.*, 2012). The ubiquity of wood frogs in environmental assessments, amphibian health monitoring, and experimental research on infectious disease, makes the species a good candidate to explore the potential benefits of incorporating hematologic assessment into study designs. No reference intervals for hematological analytes have ever been established for wild or captive wood frogs, however.

Our objectives were thus to: 1) establish hematological RI for adult wood frogs maintained in the laboratory (PCV, total RBC, WBC and thrombocyte counts, and absolute numbers of neutrophils, lymphocytes, monocytes, eosinophils, basophils and undifferentiated blast cells), 2) determine whether automated particle count flow cytometry could be reliably used as a partial substitute to the hemocytometer counting technique, and 3) investigate whether oral infection with FV3 would result in significant alterations in the hematic profile 4, 9 and 14 days post-infection.

Materials and Methods

Wood Frogs

Adult wood frogs were collected in May 2012 from an urban vernal pool (n=37) and a rural pond (n=3) in Prince Edward Island, Canada, and housed in accordance with guidelines of the Canadian Council for Animal Care for Amphibians and Reptiles (CCAC, 2004). Frogs were housed individually or as small groups in polycarbonate cages with shallow water dishes on bleach-free paper towel and fed a combination of crickets (*Acheta domesticus*), mealworms (*Tenebrio molitor*) and earthworms (Lumbricidae sp). The temperature and humidity of the room where the animals were housed were recorded daily, both as the value read immediately after entering the room in the morning, and as the minimum and maximum values of the previous 24 hours. Frogs were maintained at a relatively constant room temperature, with an average minimum-maximum of 20-22°C. Humidity varied substantially and reflected the seasonal ambient temperature (average minimum-maximum of 46-61% during acclimation and 28-44% during sampling and infection trial). A linear fluorescent bulb of UVB light (Repit-Glo 2.0, Exo-Terra, Rolf C. Hagen Inc., Montreal, QC, H9X 0A2, Canada) was placed just above the gridded cover of the tanks, 30-35 cm from the bottom, and turned on automatically for a 12-hr light:dark cycle that matched the cycle of the fluorescent lamps on the ceiling of the room. Overall health and condition of frogs were monitored daily and all mortalities that occurred in frogs housed in the same room or cage during the acclimation months were examined grossly and histologically to determine cause of death, with a particular emphasis on any lesions consistent with chytridiomycosis or suggestive of a ranavirus infection. None of the mortalities were infected with *B. dendrobatidis* or had any histological evidence of a ranaviral infection or ranaviral DNA (PCR test), so we concluded that our wild-caught adults were free of the chytrid fungus and assumed they were free of *Ranavirus* sp.

After 6 months of acclimation to captivity (October 29), frogs were moved to individual cages. A few days later, blood was obtained by puncture of the maxillary vein and collected in one, sometimes two, heparinized capillary tubes (Figure 1) (Forzán MJ *et al.*, 2012). Total volume collected depended on the ease of collection and varied from approximately 60-160 µL (from an incompletely-filled capillary tube to two full capillary tubes). Immediately after collection, 13 randomly selected frogs were orally infected with $10^{4.43}$ plaque-forming units of FV3 (Granoff A *et al.*, 1966), a dose considered lethal to adult wood frogs (Forzán MJ *et al.*, 2015). The infected frogs were euthanized 4 (n=5), 9 (n=5) or 14 (n=3) days post-infection (dpi) by submersion in a 0.1-0.2% solution of tricaine methanesulfonate (TMS, Syndel Laboratories LTD, Canada); 7 non-infected controls were killed over a period of time between 6 hours and 14 dpi. Just before euthanasia, a post-infection blood sample was collected. Infected and control frogs were examined grossly and their tissues examined histologically after euthanasia to confirm that the infected frogs had lesions consistent with FV3 infection and that control frogs were disease-free. Methodology followed a protocol approved by the Animal Care Committee of the University of Prince Edward Island (UPEI, 12-014, 6004702). Ranavirus infection was confirmed in all 13 frogs by the presence of some or all of the characteristic histopathologic lesions in the species: dermal erosion and haemorrhages; haematopoietic necrosis in bone marrow, kidney, spleen and liver; and necrosis in renal glomeruli, tongue and gastrointestinal tract mucosa (Forzán MJ *et al.*, 2015). Ranavirus DNA (PCR, primers 5'-GACTTGGCCACTTATGAC-3’ and 5' -GTCTCTGGAGAAGAAGAA-3’) (Mao J *et al.*, 1997) was also present in the liver and kidney of all frogs killed on 9 and 14 dpi and two of the frogs killed 4 dpi. All control frogs were free of infection as evidenced by negative PCR and a lack of histologic lesions.

Blood Sample Processing

Immediately after collection, two smears were made, a fixed amount of blood was transferred via a graded pipette to a pre-measured volume of Natt-Herrick solution (Natt MP & Herrick CA, 1952), and, when sufficient volume was available, the capillary tube was spun down (Hematokrit 210 centrifuge for 5 minutes at 10,000 g) to determine the PCV with a Micro-Hematorcrit capillary tube reader.

WBC differential counts

Blood smears were air-dried and within a few hours, stained automatically with Wright-Giemsa stain (Bayer HEMA-TEK 2000 Slide Stainer, Seimens, Oakville, ON, Canada). A differential cell count to determine the percentage of each WBC type was performed on the best of the two smears by counting 200 cells following standard protocols (Lassen ED & Weiser G, 2006). All differential counts were performed by one of the authors (MF) under the supervision of an experienced clinical pathologist and diplomate of the American Society for Veterinary Clinical Pathology (CG).

Total Cell Counts by Hemocytometry

A pre-determined volume of heparinized blood (20 µL and 10 µL in pre- and post-infection sampling, respectively) was mixed with 1.98 ml of Natt-Herrick solution (Natt MP & Herrick CA, 1952; Campbell TW & Ellis CK, 2007) and gently inverted several times. The mixture was refrigerated (4°C) and 19 months later, hemocytometer and automated cell counts were performed (only mild to negligible cellular deterioration occurred during this time). Hemocytometry calculated the numbers of RBC, WBC and thrombocytes using a light microscope and following a slightly modified standard methodology (Campbell TW & Ellis CK, 2007; Lassen ED & Weiser G, 2006). Briefly, the RBCs were counted in the central and four corner small squares part of the central large square, WBCs in all nine large squares, and thrombocytes in the center large square (Campbell TW & Ellis CK, 2007). To ensure no cell was counted twice, cells touching the right and lower edge of the boxes were counted and cells touching the upper and left edge were not included. Both grids on the hemocytometer were counted and an average obtained; although the discrepancy between WBC counts from the two grids was sometimes >15%, the counting procedure was not repeated (Maxham LA *et al.*, in press). Calculation of the concentration of each cell type per L of blood used the following formulas adapted from published protocols (Campbell TW & Ellis CK, 2007): WBCs (x 10^{9}/L): Number counted X 0.111 (x 0.222 for 10 µL samples), RBCs (x 12^{12}/L): Number counted X 0.005 (x 0.01 for 10 µL samples), Thrombocytes (x 10^{9}/L): Number counted X 1 (x 2 for 10 µL samples). The total concentration of each leukocyte type was calculated by multiplying its percentage from differential count by the total WBC concentration (10^9/L).

Automated Cell Counts

The blood diluted in Natt-Herrick solution that was used for hemocytometry was also used to obtain complete cell counts with an automated particle counter (Veterinary Hematology Analyzer, Sysmex XT-2000*i*V, Sysmex Canada Inc., Ontario, Canada) and corrected for the 1:100 or 1:200 dilution. Like the hemocytometry counts, automated counts were performed 19 months post-collection. Results from the automated counts were compared to the sum of all cell types (RBC, WBC and thrombocytes) calculated based on hemocytometer (manual) counts.

Statistical Analysis

All analytes were tested for normality using the Shapiro-Wilk normality test. If normally distributed (p value > 0.05), RIs were calculated using the parametric Gaussian method (mean ± 1.96 SD); if not normally distributed (p value < 0.05), RIs were calculated non-parametrically (2.5^{th}-97.5^{th} percentiles) (Friedrichs KR *et al.*, 2012). In normally distributed analytes, values outside of Tukey's interquartile fences (1.5*IQR) were considered outliers and eliminated from the analysis. In analytes that required non-parametric methods, which are less influenced by the presence of potential outliers, no values were excluded (Friedrichs KR *et al.*, 2012). Agreement between hemocytometer and automated total cell counts was established using a concordance correlation coefficient (CCC) (Lin L, 1989). Effect of ranavirus infection was assessed by qualitatively comparing the analyte values on the day of euthanasia (4, 9 or 14 dpi) with the calculated RIs, and by comparing analytes of each individual frog before infection and on the day of euthanasia using a Wilcoxon matched-pairs signed-ranks test.

Results

Morphology

Erythrocytes of wood frogs, as in other amphibians, were oval with moderate amounts of pink cytoplasm and a central ovoid densely purple nucleus often with irregular margins (Figure 2). The size of wood frog erythrocytes was similar to those of Northern leopard frogs, *Rana pipens* (Rouf MA, 1969), and larger than those of African clawed frogs (*Xenopus laevis*), African tropical clawed frogs (*X. tropicalis*), common green tree frogs (*Litoria caerulea*) and white-lipped tree frogs (*Litoria infrafrenata*) (Table 1, Figure 3) (Hadji-Azimi I *et al.*, 1987; Maxham LA *et al.*, in press; Young S *et al.*, 2012).

Identification of WBC was based on general guidelines (Weiser G & Thrall MA, 2006). Polymorphonuclear leukocytes were similar to those in other vertebrates. Neutrophils varied in size but all had typical purple multilobed to hyposegmented nuclei surrounded by clear to indistinctly-stained pale blue cytoplasm. Their cytoplasm sometimes contained minute single to multiple ill-defined blue structures (Döhle-like bodies). Eosinophils were easily identifiable by their numerous distinct round pink to orange granules. The nucleus of eosinophils, sometimes partially obscured by the cytoplasmic granules, was purple and less segmented than that of neutrophils. Basophils were usually smaller than eosinophils, with a cytoplasm filled with deeply purple granules that usually completely obscured the nucleus (Figure 2).

Identification of mononuclear leukocytes was more difficult. The morphology of lymphocytes varied the most. Most lymphocytes were smaller than neutrophils, had a round nucleus with densely clumped chromatin and scant blue cytoplasm; the cytoplasmic membrane of these small lymphocytes was often undulated, and their shapes slightly molded to accommodate surrounding RBCs. Slightly less abundant were large lymphocytes, with round, ovoid or slightly indented nuclei and abundant pale blue cytoplasm, resembling those found in some ruminants (Weiser G & Thrall MA, 2006). Occasionally, the large lymphocytes had a pale, almost clear, cytoplasm with a few minute round pink granules (granular lymphocytes). Plasmacytoid lymphocytes were only rarely observed. Monocytes were mostly as large or larger than large lymphocytes, with an ovoid to reniform (rarely pseudo-lobulated) nucleus with lacey or loosely clumped chromatin surrounded by moderate to abundant grey-blue cytoplasm, often with small clear vacuoles (Figure 2). Undifferentiated blast-like cells (henceforth referred to as blasts) varied in size between the lymphocytes and monocytes, had a small amount of dark blue cytoplasm, and a large round nucleus with coarsely clumped chromatin and occasionally distinct single round nucleoli.

Thrombocytes were mostly small and round, with a very dark nucleus which chromatin was particularly dense at its core, a ragged cytoplasmic membrane, and little pale to clear cytoplasm that occasionally contained a red-magenta vacuole (activated thrombocytes (Claver JA & Quaglia AIE, 2009; Tanizaki Y *et al.*, 2015)) (Figure 2). Elliptical (non-activated) thrombocytes were less frequently observed; they had a dark ovoid nucleus which chromatin was dark and most dense along the nuclear long axis. Cytoplasm in elliptical thrombocytes was very pale blue to clear, and the cytoplasmic membrane was smooth, sometimes tapered at the edges, giving the cell a fusiform shape (Figure 4).

When cytochemical stains were applied (Bricker NK *et al.*, 2012), only the cytoplasm of neutrophils and eosinophils, and some granules in basophils were positively stained with Periodic Acid Schiff (PAS) (Figure 2). Sudan Black failed to stain neutrophils, eosinophils, basophils, lymphocytes and thrombocytes; staining characteristics of monocytes could not be established because of their paucity in the slide examined.

Hematology

Proposed RIs are presented in Table 2. Shapiro-Wilk normality tests showed that except for percentage and absolute number of eosinophils, percentage of basophils, and absolute monocyte counts, all analytes were normally distributed (p value > 0.05).

RBC analytes of wood frogs were compared to reported values for other species of frogs (Table 1, Figure 3). The percentage of the total cell count represented by the RBC was similar in all frog species. PCV and RBC counts in wood frogs were similar to those of Northern leopard frogs (Rouf MA, 1969), but lower than those of Australian tree frogs (*Litoria caerulea* and *L. infrafrenata*) (Young S *et al.*, 2012) and African clawed frogs (*X. tropicalis* and *X. laevis*) (Hadji-Azimi I *et al.*, 1987; Maxham LA *et al.*, in press).

Of the leukocytes, lymphocytes were found in the highest abundance, followed by basophils and neutrophils, in that order; monocytes, eosinophils and blasts were equally uncommon (Table 2).

Hemocytometer (Manual) and Automated Total Cell Counts

Results are based on paired samples from 38 out of the 40 wood frogs sampled to calculate the RIs (one frog yielded insufficient blood for absolute counts, another frog was eliminated as an outlier based on Tukey's interquartile fences). There was mild cellular deterioration evidenced by occasional loss of cytoplasmic definition in erythrocytes and occasional cytoplasm blebs pinched off poorly preserved cells (Figure 4). Distinguishing small WBC from activated thrombocytes was often difficult. The vast majority of cells, as calculated by hemocytometry, were RBC (96.3%, 95% CI 93.1-99.4); WBC (1.9%, 95% CI 0.3-3.1) and thrombocytes (1.9%, 95% CI 0.3-3.5) comprised only a small proportion. The CCC comparing hemocytometer total cell counts (considered the gold standard) to automated (particle counter) total cell counts was 0.845 (95% CI 0.753-0.938, n=38), indicating a "substantial to almost perfect agreement" between methods, according to the nomenclature proposed by Landis & Koch (Landis JR & Koch GG, 1977) (Figure 5).

Effect of Infection with FV3

At the time of euthanasia, wood frogs infected with FV3 had reduced total cell counts (manual 10/12 frogs; automated 9/12 frogs), RBC counts (10/12 frogs) and basophil percentage (11/13 frogs), and increased neutrophils (percentage 13/13 frogs; absolute count 10/12 frogs) and blasts (percentage 8/13 frogs; absolute count 8/12 frogs). Lymphocyte percentage was reduced in frogs killed 4 (5/5 frogs) and 9 (4/5 frogs) dpi but increased in frogs killed 14 dpi (2/3 frogs); no pattern was found in absolute numbers of lymphocytes. No trend was evident in the monocyte and eosinophil counts. Most changes observed when comparing pre and post-infection analytes for each individual frog were statistically significant (p value < 0.05, see Table 3). Post-infection analytes of some of the frogs that exhibited a change from their pre-infection values were also outside of the proposed RIs (Table 3). Thrombocyte counts were not considered in the comparisons because of their great variability and how affected they were depending on the presence or absence of clumping.

Intracytoplasmic inclusions, likely of viral origin, were observed in blood cells of frogs infected with FV3 and euthanized 9 and 14 dpi. These deeply pink to red glassy homogeneous to granular inclusions measuring 2-9 µ were present in the following cells (listed in order of frequency): lymphocytes, neutrophils, monocytes, eosinophils and blasts. Coinciding with the appearance of intracytoplasmic inclusions was the presence of nuclear deterioration and mild toxic changes in neutrophils and alterations in the morphology of most other blood cells (Figure 6).

There was a near perfect agreement between the hemocytometer (manual) and automated cell counts involving only the 13 infected and 7 control frogs prior to infection (CCC = 0.903) but only fair to moderate agreement in samples from those frogs taken at the time of euthanasia (CCC = 0.417) (Landis JR & Koch GG, 1977).

Discussion

As expected, the morphology of leukocytes in wood frogs was similar to that of mammals and, in general terms, other amphibians (Campbell T, 2006; Claver JA & Quaglia AIE, 2009; Weiser G & Thrall MA, 2006). Based on our rather conservative cell classification and identification, blood cells in wood frogs include erythrocytes, thrombocytes, lymphocytes, monocytes, neutrophils, eosinophils and basophils. Neutrophils often contain small structures resembling mammalian Döhle bodies, also reported in healthy African clawed and Australian tree frogs (Maxham LA *et al.*, in press; Young S *et al.*, 2012). We found no evidence of granulocytes that resembled heterophils, which have rod-shaped orange-pink granules and have been reported in some amphibian species. Undifferentiated blast-like cells of uncertain lineage are often present, albeit in small numbers. Amphibians are, of course, a large class of vertebrates that includes approximately 6,000 species, so morphological identification of blood cells is far from standardized. For instance, cells that we have called granular lymphocytes resemble what other researchers may have called azurophils (Claver JA & Quaglia AIE, 2009). Lymphocytes appear to be the most numerous WBC present in wood frog blood, resembling what has been reported in at least two other frogs in the Ranidae family: the green frog (*Rana clamitans*) (Fleming TB, 2011) and the Northern leopard frog (*Rana pipiens*) (Rouf MA, 1969). Conversely, the bullfrog (*Rana catesbeiana*), although also a ranid frog, has a predominance of neutrophils (Coppo JA *et al.*, 2005), as does the African clawed frog (*X. tropicalis*), a member of the Pipidae family (Maxham LA *et al.*, in press). As with other amphibian species (Allender M & Fry M, 2008; Maxham LA *et al.*, in press), distinguishing between large lymphocytes and monocytes was difficult. The two special stains used (PAS and Sudan Black) were of no practical use in cellular identification. The PAS stain was effective in highlighting neutrophils and basophils, but those cells are already easily identifiable with routine Wright-Giemsa. We can confidently note that neutrophils, basophils, eosinophils, lymphocytes and thrombocytes do not pick up the Sudan Black stain. Because monocytes are so rare in wood frogs, we could not determine whether they were positive or negative for Sudan

Black, so this stain was also of no practical use in wood frogs. Other cytochemical or immunocytochemical stains may improve our ability to properly identify mononuclear leukocytes in amphibians (Bricker NK *et al.*, 2012). Unfortunately, such stains are rarely used or have not been standardized for use on wood frogs, to the authors' knowledge. Using the few reports on amphibian hematology for a qualitative comparison, the percentage of RBC in the total blood cell count of wood frogs is similar to those of other frog species. Other RBC analytes in wood frogs are similar to those of another frog of the Ranidae family, but different from frogs in the Hylidae (tree frogs) and Pipidae (African clawed frogs) families. Frogs in the Ranidae family have larger erythrocytes and a lower RBC count than hylidae or pipidae frogs (Maxham LA *et al.*, in press; Young S *et al.*, 2012). The inverse association between erythrocyte size and RBC count observed in wood frogs has been found in other species (e.g., *X. tropicalis*, *Rana esculenta* and *Rana temporaria*) (Maxham LA *et al.*, in press; Schermer S, 1967): the larger the size of the erythrocytes, the smaller their numbers. PCV is also associated with erythrocyte size, so that frogs with small erythrocytes have higher PCVs. The relation of PCV to erythrocyte size is not linear but curvilinear, indicating that at some point PCV will no longer increase even if a certain frog species has even smaller erythrocytes. These comparisons are only qualitative and do not support definitive conclusions. However, the trends observed suggest differences in RBC characteristics associated with the evolutionary classification and life history of a given species or family.

Although total cell counts were performed several months after collection and there was evidence of occasional cellular disintegration, samples collected and fixed in Natt-Herrick solution have been found adequate for hemocytometry and automated counts for up to two years after collection (Maxham LA *et al.*, in press). The difficulty in distinguishing WBCs from thrombocytes in the hemocytometer chamber could have been worsened by the time lapsed between collection and counting, but it was more likely due to their size and shape similarities, particularly as most thrombocytes were activated and thus round rather than elliptical. In support of the validity of our results, even with the difficulties encountered in identifying each cell type, we have the high agreement between manual (hemocytometer) and automated total cell counts. This agreement between the gold standard (manual) and automated counts also suggests that automated cell counters could be used to partially replace the manual counts, as suggested for *X. tropicalis* (Maxham LA *et al.*, in press) as long as the counting method is not affected by, or can be adjusted to, the particle size. Using an instrument that only counts particles larger than a specific size and cannot be adjusted risks missing cells which nuclei are below that threshold (Whittington RJ & Comer DAM, 1984). The RBC count would be obtained by subtracting the WBC+thrombocyte count from the automated total cell count and adjusting for the appropriate dilution. Although the agreement between hemocytometry and automated counts was higher in wood frogs than what is reported for *Xenopus tropicalis*, the difference between counts did not follow an obvious trend, as it did with *X. tropicalis* (Maxham LA *et al.*, in press). This apparent increased variability in wood frogs could be a reflection of the heterogeneity of our experimental subjects, all of which were wild-caught and assumed to be more genetically varied than laboratory-bred *X. tropicalis*. Repeated studies would be needed to determine if this is a true difference or a casual finding associated with sampling or counting methods. For instance, thrombocyte clumping was often present in the wood frog samples, perhaps as a consequence of the collection method employed. The individual counting of thrombocytes, even when clumped, would have increased our counting error and affected the total cell counts since clumping would have altered the homogeneous distribution of cells in suspension.

Contrary to those samples used to calculate the RIs and discussed above, samples collected at the time of euthanasia showed only fair to moderate agreement between manual and automated total cell counts. Poor agreement occurred both in the frogs infected with FV3 and those used as controls, so it was not associated with infection status. This puzzling outcome, so different to what was observed in samples used to calculate the RIs, is almost certainly associated with the slight but important change in collection method. When sampling on the day of euthanasia, the amount of blood diluted in Natt-Herrick solution was half of what was used for the calculation of RIs (10 μL instead of 20μL) but the amount of Natt-Herrick solution remained unchanged. This reduced the ratio of sample to diluent from 1:100 to 0.5:100. The change was made to ensure enough blood was collected to perform the total and differential cell counts: a smaller volume diluted in Natt-Herrick solution meant sufficient blood would be available for the blood smear and spinning down to obtain the PCV. Unfortunately, even if calculations were adjusted accordingly, the smaller sample volume resulted in a much larger variation in the results. It seems advisable to consider 20 μL of blood as the minimum necessary for hematological evaluation.

In spite of the increased variance in results from samples at the time of euthanasia, clear changes were noted in the blood profile of wood frogs infected with FV3. As FV3 infection causes necrosis of hematopoietic tissue in wood frogs (Forzán MJ *et al.*, 2015), alterations in the hematological profile were to be expected. These alterations were observed when pre- and post-infection values were compared for each frog. Less frequently, analytes post-infection were outside the proposed RIs. Although anemia was noted in infected wood frogs, the decrease in RBC counts was only statistically significant 4 dpi and was also observed in some control frogs (data not shown). The anemia may have simply been due to the previous bleeding and not associated with infection. Changes in the leukocytes are more likely to be directly associated with the infection. A relative lymphopenia was present in wood frogs infected with FV3 on dpi 4 and 9, but at 14 dpi a relative lymphocytosis was noted. The early lymphopenia could have been a reflection of a stress response, particularly as it was accompanied by neutrophilia (Davis AK *et al.*, 2008), the result of lymphocytes migrating to sites of viral replication, or a viral-induced reduction (Allender MC & Mitchel MA, 2013). The later lymphocytosis suggests an active immune response to the virus. Absolute and relative neutrophilia was present at all sampling times post-infection, and thus a response to the viral infection rather than a simple stress response. To our knowledge, the only other study evaluating hematological profiles after infection with a *Ranavirus* sp (FV3-like) was performed in a reptile: the red-eared slider turtle, *Trachemys scripta elegans* (Allender MC & Mitchel MA, 2013). Although no significant alterations in the blood cell counts were noted in the infected turtles possibly because of a small sample size, a trend towards lymphopenia was noted.

Whether the changes in the hematological profiles of infected frogs represent an active immune response to the virus is difficult to establish. It is hard to explain an increase in any circulating leukocyte when, considering the hematopoietic necrosis caused by FV3 in wood frogs (Forzán MJ *et al.*, 2015) and by Bohle-like ranavirus in tree frogs, *Litoria splendida* and *L. caerulea* (Jerrett IV *et al.*, 2015), immunosuppression would intuitively be expected. Perhaps infection results in necrosis of a particular cell type rather than all hematopoietic precursors, and thus allows for increases in other cells. Or it could be that infection initially stimulates a proliferation of hematic cells that is later followed by degeneration and necrosis. Further studies into the pathogenesis of FV3 infection in wood frogs may help explain this apparent paradox, but it will also be necessary to determine whether a certain hematologic profile accurately reflects immune competence. One such study has been conducted to evaluate the response of tree frogs, *Litoria caerulea* and *L. infrafrenata*, to infection with *B. dendrobatidis* (Young S *et al.*, 2012). The authors found that the decrease in WBC counts associated with *B. dendrobatidis* infection is also associated with a decreased lymphocytic response to antigenic stimulation. Similar studies need to be performed to establish what effect FV3 and other *Ranavirus* spp. have on the immune system of wood frogs.

Evidence of infection of wood frog hematic cells by FV3 is supported by the presence of the intracytoplasmic inclusions observed in the circulating blood cells. Similar inclusions, known to correspond to viral assembly sites, are reported in other species (e.g. Allender MC & Mitchel MA, 2013). Intriguingly, a “peculiar, brilliant red, oval body” in the cytoplasm of “degenerative” neutrophils, strikingly similar to the inclusions found in our wood frogs, is described in a mid-20th century German textbook in reference to apparently healthy hibernating ranid frogs (Schermer S, 1967). Either *Ranavirus* sp. has infected European frogs much longer than more recently reported mortalities suggest, or the inclusions reflect cytoplasmic changes associated with non-specific cellular deterioration, but not actual sites of viral replication. Regardless, as inclusions were only seen in deteriorating cells, and deteriorating cells were only present in infected frogs at the latest stages of infection, we can infer an association between inclusions and FV3 infection.

Our findings indicate that the hematological profile of wood frogs is altered by infection with FV3 and that, albeit less likely, changes can sometimes be detected by comparing results to proposed RIs. The RIs proposed here could be used to evaluate the health of captive wood frogs, but great care must be taken if they are to be used in evaluation of free-ranging individuals, as the environment has a large influence on amphibian blood composition (e.g. Weathers W, 1975). We describe alternations in the hematological profile associated with a fatal infection with FV3. Our findings, although preliminary, suggest that adult wood frogs are capable of mounting an immune response to FV3 infection, and that the response involves cells of the adaptive immune system, namely circulating lymphocytes. Further studies will determine whether an immune response can indeed be induced and whether prophylactic immunization would be effective in protecting amphibians against subsequent exposure to this emerging pathogen.

Acknowledgments

We would like to thank Dr. Tricia Fleming and Ms. Anne Dover for their indispensable mentoring on hematologic techniques, Mr. Alfred Mitchell for performing the automatic cell counts, Mr. Allan MacKenzie for preparing the Natt-Herrick solution and assisting us with hemocytometer calculations, Dr. Shannon Martinson for photographing the sampling procedure, and most specially Ms. Kathleen Jones without whose help the experimental infection trial would not have been possible. Funding was provided by an Alexander Graham Bell Graduate Scholarship-Doctoral (Canadian Natural Science and Engineering Research Council), the Canadian Cooperative Wildlife Health Centre (now Canadian Wildlife Health Cooperative) and Environment Canada (Bruce Pauli).

Table IV - 1. Comparison of erythrocyte size, RBC count, PCV, and percentage of RBC in the total cell counts of wood frogs (*R. sylvatica*) with other anuran species: Northern leopard frog (*R. pipiens*) (Rouf 1969), White's tree frog (*L. caerulea*), White-lipped tree frog (*L. infrafrenata*) (Young 2012), African tropical clawed frog (*X. tropicalis*) (Maxham, *in press*) and African clawed frog (*X. laevis*) (Hadji-Azimi 1987).

Family	Species	Common name	Size (µ)	RBC x 10^{12}/L[a]	PCV (%)[b]	RBC (%)
Ranidae	*Rana sylvatica*	Wood frog	**25 x 18.5**	**0.45(0.3-0.6)**	**30(19-41)**	**96**
	Rana pipiens	Northern leopard frog	24 x 17	0.32(0.2-0.5)	25(6-44)*	96
Hylidae	*Litoria caerulea*	White's tree frog	19 x 12	0.7(0.4-1.0)	36(23-48)	94
	Litoria infrafrenata	White-lipped tree frog	18 x 11	0.8(0.4-1.1)	34(19-49)	93
Pipidae	*Xenopus tropicalis*	African tropical clawed frog	13 x 10	1.5(1.0-2.0)	41(27-54)	98
	Xenopus laevis	African clawed frog	17.5 x 10.5	0.8(0.5-1.1)	37(19-55)	97

[a] Average (RI), [b] Average (95% CI), (*) hematocrit, NA = not available.

Table IV - 2. Hematological analytes of adult *Rana sylvatica*. Reference intervals (RI) were established as mean ± 1.96 standard deviation (normally distributed data) [a] or 2.5th-97.5th inter-quantile range (non-normally distributed data)[b] after outliers were eliminated (outside of Tukey's interquartile fences); distribution normality was established by Shapiro-Wilk test (*P* value >0.05 = normal distribution). RI for three other species in the Ranidae family, green frogs (*R. clamitans*) (n=59-61, wild-caught) (Fleming 2011), bullfrogs (*R. catesbeiana*) (n=302, captive-bred) (Coppo 2005, captive bred) and Northern leopard frogs (*R. pipiens*) (n=12-56, wild-caught) (Rouf 1969), are similarly calculated based on published literature and provided for ease of comparison as mean(RI). RI values under zero are reported as 0 or * if zero would be incompatible with life.

Analyte	**n**	**Mean**	**SD**	**Median**	**Min**	**Max**	***P* value**	**RI (95%CI)**	***R. clamitans***	***R. catesbeiana*[a]**	***R. pipiens***
PCV (%)	26	29.5	5.6	29.0	20.0	41.0	0.422	**18.6-40.5**[a]	35.8(23.0-48.5)	30.1(19.3-40.9)	
RBC (10^{12}/L)	38	0.41	0.08	0.42	0.21	0.56	0.945	**0.25-0.57**[a]	0.34(0.19-0.49)	0.42(*-1.82)	0.32(0.16-0.48)
Thrombocyte (10^{9}/L)	38	8.3	3.7	8.0	2.0	16.5	0.642	**1.3-15.2**[a]	14.0(1.6-26.3)		7.3(1.1-13.5)
WBC (10^{9}/L)	38	7.65	2.68	7.27	3.0	13.9	0.315	**2.2-13.1**[a]	8.3(2.2-14.5)	20.5(11.3-29.7)	5.5(0.7-10.3)
Lymphocyte (%)	39	76.85	6.69	76.50	64.0	90.0	0.698	**63.7-90.0**[a]	67.4(25.5-87.8)	26.8(17-36.6)	53.4(23.8-83.0)
Lymphocyte (10^{9}/L)	38	5.76	2.28	5.30	1.96	10.17	0.109	**1.3-10.2**[a]	9.5(0.7-24.3)		
Neutrophil (%)	39	6.83	3.29	6.50	2.00	14.50	0.098	**0.4-13.3**[a]	12.0(2.0-43.5)	60.9(36.1-85.7)	26.5(3.7-49.3)
Neutrophil (10^{9}/L)	35	0.46	0.23	0.42	0.09	1.11	0.084	**0-0.9**[a]	1.4(0.1-4.6)		
Monocyte (%)	34	1.64	0.09	1.50	0	3.50	1.000	**0-3.4**[a]	4.1(0-25.1)	2.9(0.7-5.1)	11.0(1.4-20.6)
Monocyte (10^{9}/L)	34	0.13	0.09	0.12	0	0.42	0.023	**0-0.35**[b]	0.5(0-3.3)		
Eosinophil (%)	40	1.55	1.3	1.0	0	4.5	0.004	**0-4.5**[b]	13.8(2.0-41.5)	5.8(2.6-9)	7.3(3.7-49.3)
Eosinophil (10^{9}/L)	35	0.09	0.07	0.08	0	0.28	0.020	**0-0.25**[b]	1.8(0.1-6.8)		
Basophil (%)	33	10.65	2.52	11.00	5.50	16.00	0.023	**5.9-14.8**[b]	2.7(0-10.5)	3.5(1.1-5.9)	4.4(0-10.6)
Basophil (10^{9}/L)	38	0.80	0.34	0.78	0.16	1.46	0.496	**0.1-1.5**[a]	0.4(0-3.3)		
Blast (%)	39	1.47	0.87	1.50	0	3.50	0.323	**0-3.2**[a]			
Blast (10^{9}/L)	38	0.11	0.07	0.10	0	0.26	0.063	**0-0.3**[a]			

Table IV - 3. Hematological analytes of adult *Rana sylvatica* orally infected with $10^{4.43}$ plaque-forming units of Frog Virus 3 and euthanized 4 (n=5), 9 (n=5) or 14 (n=3) days post-infection (dpi). Significance (p value < 0.05, Wilcoxon matched-pairs signed-ranks test comparing pre and post-infection results from all 13 frogs) is noted as an upward or downward arrow, indicating increase or decrease of the analyte post-infection; non-significant trends are indicated by a diagonal arrow; an empty cell indicates no difference. The number of frogs in each group with values outside the proposed reference interval (RI, as per Table 2) is indicated if >0 (O-RI)

Analyte	**4 dpi (n=5)**			**O-RI**		**9 dpi (n=5)**			**O-RI**		**14 dpi (n=3)**			**O-RI**		**p value**	**RI**
	Mean (SD)	Med	Min-Max			Mean (SD)	Med	Min-Max			Mean (SD)	Med	Min-Max				(95%CI)
PCV (%)	31.4(3.5)	32.5	26.0-35.5			25.7(2.5)	26.6	22.3-28.3			*	*	*				18.6-40.5
Erythrocyte (10^{12}/L)	0.27(0.11)	0.25	0.16-0.40	3	↓	0.33(0.4)	0.32	0.29-0.38			0.39(0.8)	0.44	0.30-0.45			0.019	0.25-0.57
Thrombocyte (10^{9}/L)	15.4(12.0)	8.0	5.0-29.0			3.6(2.2)	3.0	1.0-7.0			3.0 3.6)	2.0	0.0-7.0				1.3-15.2
Leukocyte (x 10^{9}/L)	8.3(4.6)	7.1	3.3-15.8			8.5(2.1)	7.9	6.2-11.5			9.7(3.4)	8.1	7.4-13.7				2.2-13.1
Lymphocyte (%)	71.5(6.2)	73.5	61.5-77.0	1	↓	72.2(17.0)	77.0	43.0-87.0	1	↓	70.0(15.2)	77.5	53.0-80.0	1	↑	0.050[a]	63.7-90.0
Lymphocyte (10^{9}/L)	6.0(3.3)	5.3	2.1-11.0			6.8(2.5)	6.3	4.2-10.0			6.7(3.7)	5.5	3.8-10.9				1.3-10.2
Neutrophil (%)	12.5(4.2)	10.5	10.0-20.0	1	↑	18.6(17.9)	11.5	5.5-50.0	2	↑	19.3(11.2)	17.0	10.0-32.0	3	↑	0.005	0.4-13.3
Neutrophil (10^{9}/L)	0.9(0.4)	0.8	0.7-1.7	2	↑	1.8(1.8)	0.9	0.6-4.9	2	↑	1.6(0.6)	0.3	1.2-2.3	3	↑	0.008	0-0.9[a]
Monocyte (%)	2.0(2.3)	1.0	0.0-4.0			1.4(1.8)	1.0	0.0-5.0			1.7(2.5)	0.5	0.0-5.0				0-3.4
Monocyte (10^{9}/L)	0.1(0.2)	0.0	0.0-0.4			0.1(0.1)	0.1	0.0-0.4			0.1(0.2)	0.0	0.0-0.3				0-0.35
Eosinophil (%)	2.1(1.7)	2.0	0.5-5.0			0.7 (0.6)	0.5	0.5-2.0			1.0(0.5)	1.0	1.0-2.0				0-4.5
Eosinophil (10^{9}/L)	0.2(0.2)	0.1	0.03-0.3			0.1 (0)	0.1	0.0			0.1(0.1)	0.1	0.1				0-0.25
Basophil (%)	9.2(2.8)	10.0	5.0-12.0	1	↓	6.2(2.0)	5.5	4.0-9.0	2	↓	4.8(1.0)	4.5	4.0-6.0	2	↓	0.005	5.9-14.8
Basophil (10^{9}/L)	0.8(0.6)	0.7	0.3-1.9			0.6(0.2)	0.5	0.3-0.9			0.4(0.1)	0.4	0.3-0.6				0.1-1.5
Blast (%)	2.8(2.2)	2.5	0.5-5.5	1	↗	1.4(0.9)	1.5	1.0-3.0		↗	0.1(0.1)	0.1	1.0-6.0	1	↗	0.063	0-3.2
Blast (10^{9}/L)	0.3(0.3)	0.1	0.0-0.9		↑	3.3(2.6)	4.0	0.0-0.3			0.4(0.4)	0.3	0.0-0.8		↑	0.050[a]	0-0.3

*PCV could only be measured in one frog, PCV = 19.2 %. [a] Actual p value = 0.0499

Figure IV - 1. Blood collection from the maxillary vein of an adult wood frog, *Rana sylvatica*. Immediately after puncturing the skin with a 25 gauge needle, the trickling blood is collected into a heparinized capillary tube (Forzán *et al.*, 2012).

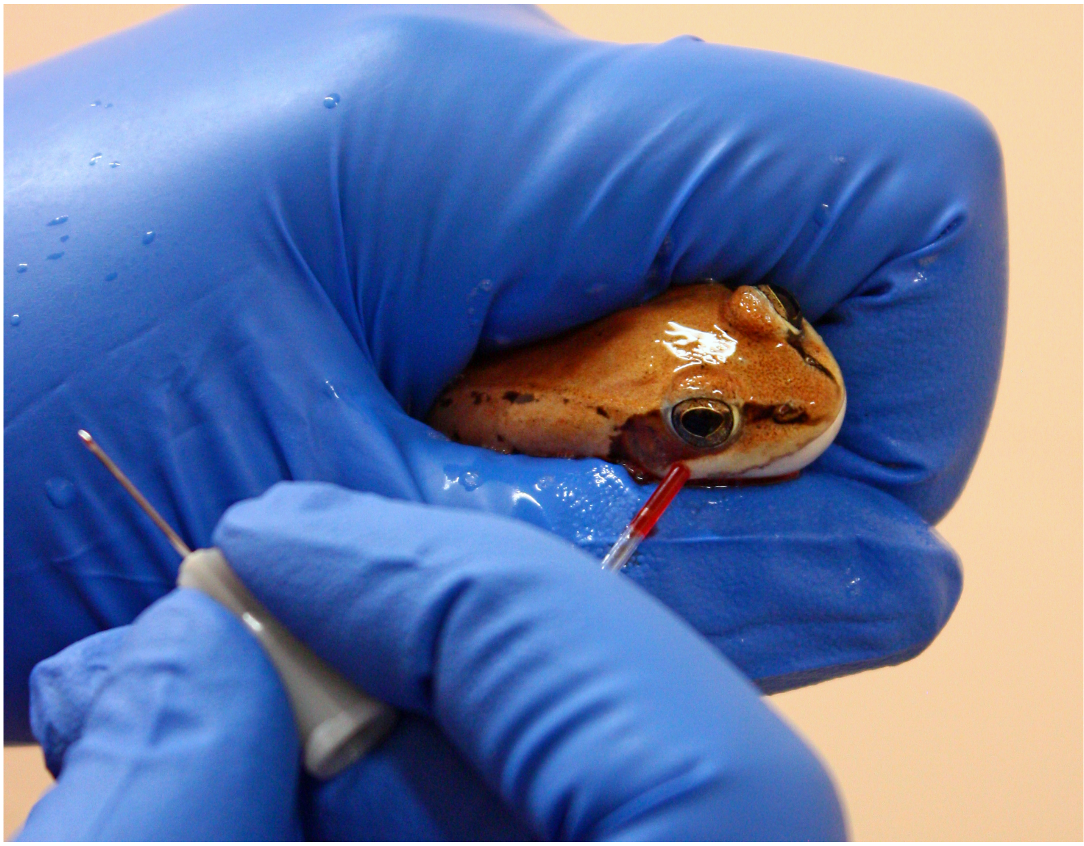


Figure IV - 2. Morphology of blood cells in wood frogs, *Rana sylvatica*. A) neutrophil, B) basophil, C) partially degranulated basophil, D) eosinophil, E) small lymphocyte, F) medium-large lymphocyte next to a neutrophil, G) medium-large lymphocyte with small cytoplasmic granules, H) large lymphocyte with intracytoplasmic granules, I) plasmacytoid lymphocyte next to an eosinophil, J) monocyte, K) unidentified blast cell, L) cluster of round (activated) thrombocytes (insert: single thrombocyte with magenta vacuole in cytoplasm); stained with Wright-Giemsa. M) neutrophil, N) basophil, O) eosinophil, P) lymphocyte and Q) thrombocyte; stained with Periodic Acid Schiff (PAS). The cytoplasm of neutrophils and eosinophils, and some of the granules in the basophils were PAS positive, while lymphocytes and thrombocytes failed to stain. Bars = 10 µm.

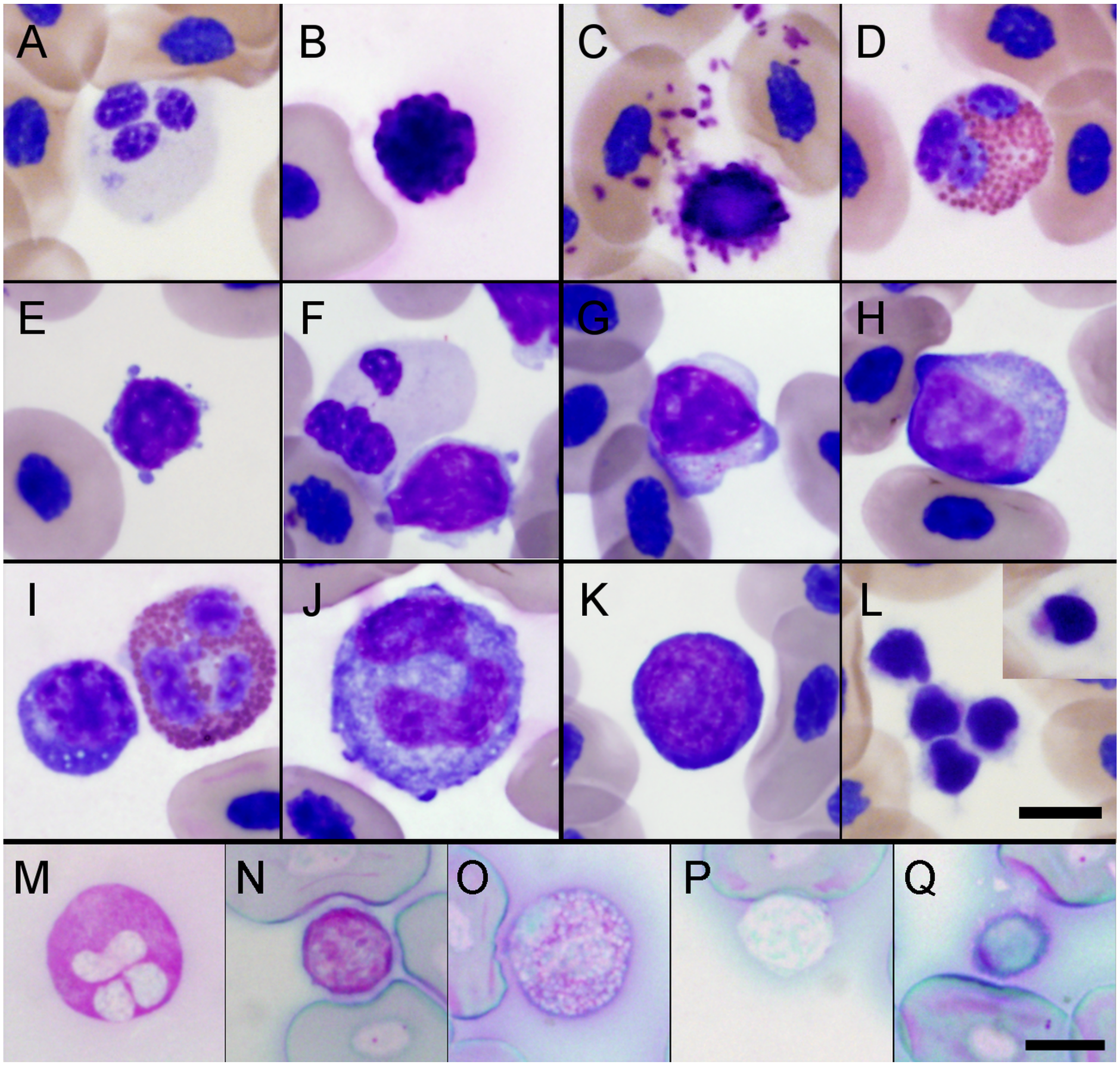


Figure IV - 3. Erythrocyte size, red blood cell (RBC) count and packed cell volume (PCV) of selected species of frogs. The ovals are scaled to reflect the average shape and size of each species' erythrocytes.

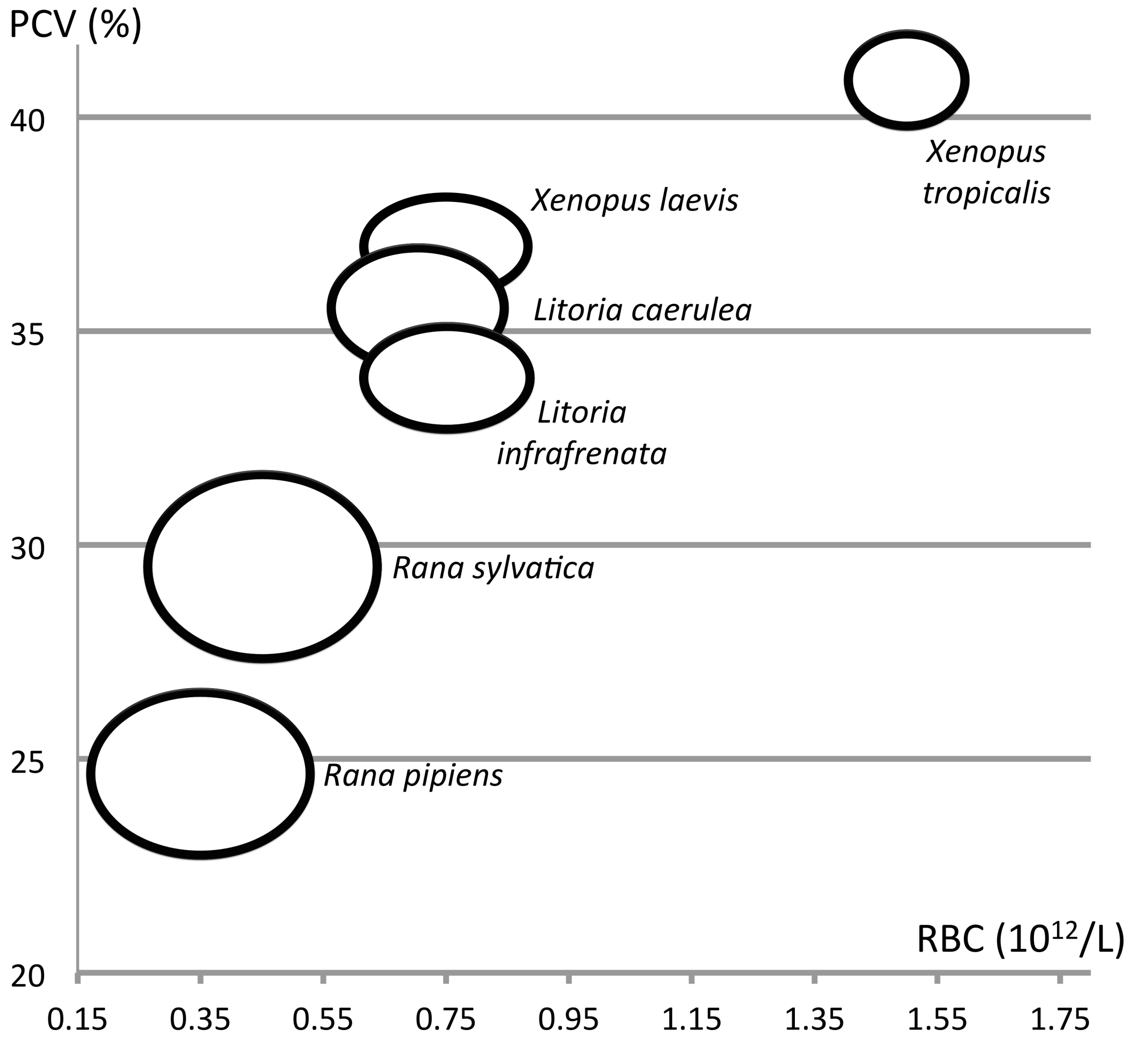


Figure IV - 4. Blood cells of wood frogs, *Rana sylvatica*, preserved in Natt-Herrick solution (Natt-Herrick 1952). Characteristic morphology of well preserved WBCs, RBCs and thrombocytes: A) granular WBCs (Bar = 40 µm, insert Bar = 10 µm), B) large RBC next to a non-granular WBC with smooth membrane contours, C) elongated thrombocyte next to slightly rounded RBC, D) activated thrombocyte with ragged membrane countour (Bars = 10 µm). E) Low magnification of hemocytometer grid (Bar = 200 µm). Atypical morphologies: F) damaged RBC amongst other well preserved erythrocytes, G) WBC cell with bilateral blebs of cytoplasm and folded RBC (Bars = 20 µm), H) clump of three activated thrombocytes adjacent to a single smooth-contoured WBC and several typical RBCs (Bar = 10 µm).

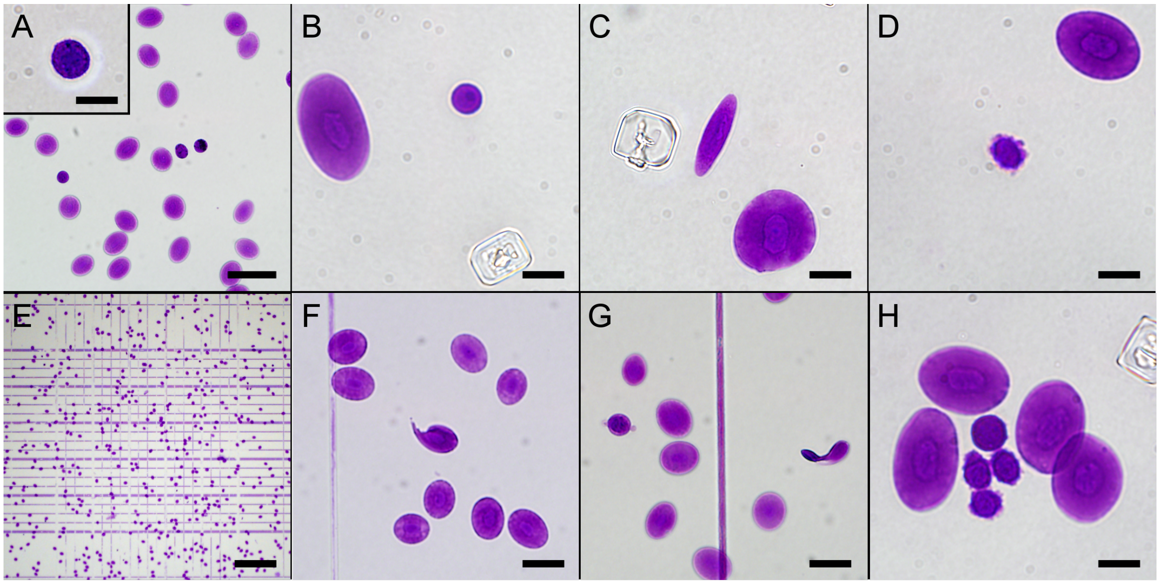


Figure IV - 5. Comparison between manual and automated blood cell counts in wood frogs, *Rana sylvatica*. Concordance correlation coefficient (CCC = 0.845) of manual (hemocytometer) versus automated total cell counts (WBC+RBC+thrombocytes, n=38). The dotted line represents perfect (expected) agreement whereas the solid line represents the observed agreement between automated and manual total cell counts. Media (95% CI): manual 432 (265-579), automated 429 (274-569).

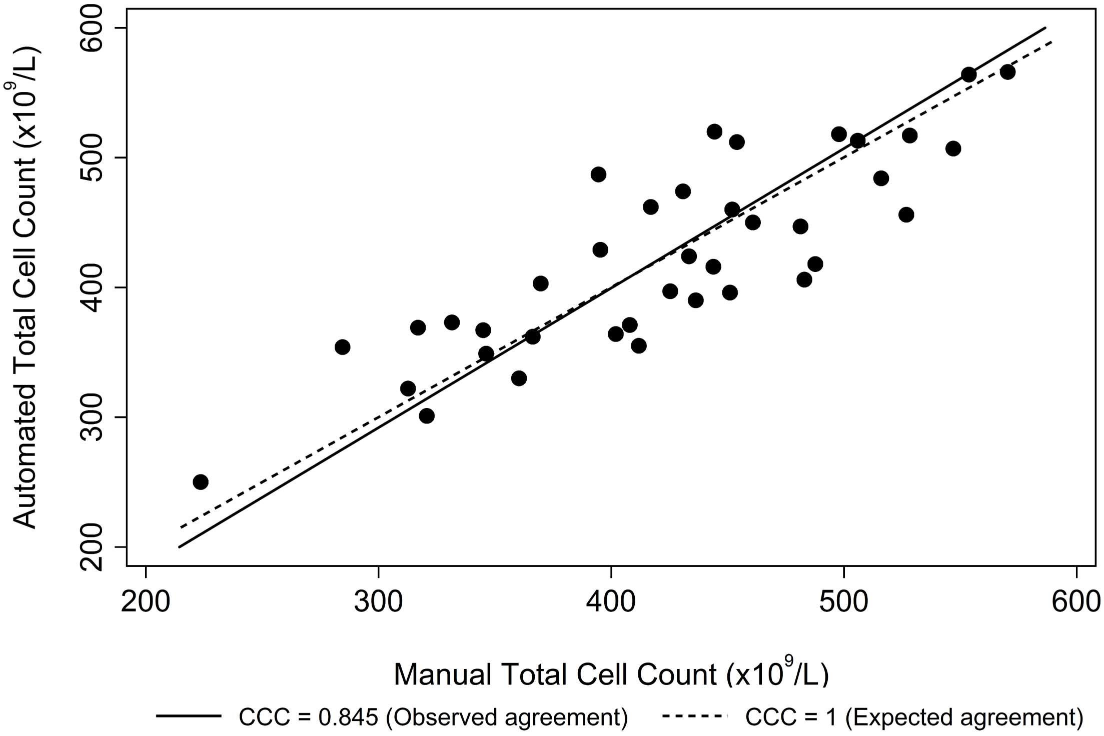
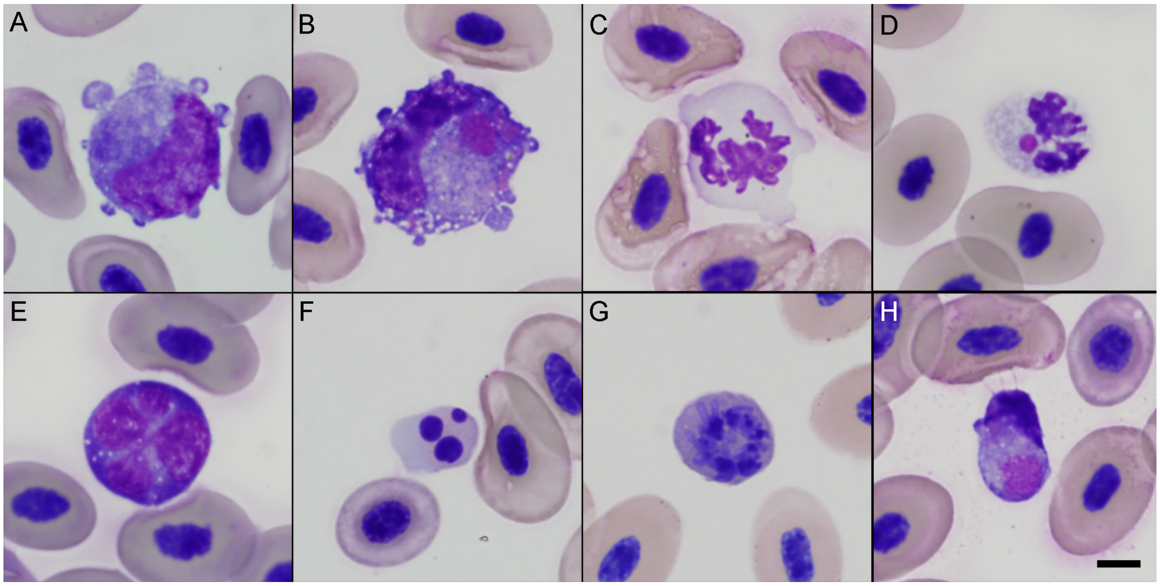


Figure IV - 6. Alterations observed in blood cells of wood frog, *Rana sylvatica*, 14 days after oral inoculation of Frog Virus 3 (FV3, *Ranavirus* sp). A) reactive monocyte, B) reactive monocyte with large pink-red cytoplasmic inclusion, C) neutrophil undergoing nuclear deterioration, D) neutrophil with nuclear deterioration, mild toxic change and intracytoplamic inclusion, E) undifferentiated blast-like cell with abnormal nuclear morphology, F) unidentified cell with karyorrhectic nucleus, G) mitotic figure (probably in RBC), H) unidentifiable cell with cytoplasmic inclusion. Intracytoplasmic inclusions are likely FV3 inclusion bodies. Bar = 10 µm.



CHAPTER FIVE

Pathogenesis of Frog Virus 3 (*Ranavirus* sp, Iridoviridae) infection in wood frogs, *Rana sylvatica* (*Lithobates sylvaticus*)

Abstract

Wood frogs, *Rana sylvatica*, are highly susceptible to infection with Frog Virus 3 (FV3), the type species of the genus *Ranavirus* (Iridoviridae) and a common cause of mass mortality in wild populations. Wood frogs, which are widely distributed in North America, have become a focus of field and laboratory research into the ecology and the host-pathogen interaction in ranavirosis. To elucidate part of the pathogenesis of FV3 infection in wood frogs, 40 wild-caught adults (averaging 53 mm in length) were acclimated to captivity for six months, inoculated orally with a fatal dose of $10^{4.43}$ pfu/frog, and euthanized at 0.25, 0.5, 1, 2, 4, 9 and 14 days post-infection (dpi). Lesions associated with FV3 infection were minimal 4 dpi, evident 9 dpi and severe 14 dpi. Viral DNA was first detected in liver 4 dpi; by dpi 9 and 14 all viscera tested (liver, kidney and spleen), the skin and feces were positive (PCR). Immunohistochemical (IHC) staining detected virus in multiple tissues (skin, bone marrow, oral/nasal cavity, tongue, blood vessels, liver, kidney, lung and digestive tract) on dpi 9 and 14 only. IHC staining was consistent with presence of histological lesions except in the spleen, where staining was absent in spite of large foci of lymphoid necrosis. Direct contact (skin) and fecal-oral contamination are likely effective routes of transmission and, thus, skin or cloacal swabs can be sources of ante-mortem diagnostic samples, but only in late stages of disease (9-14 dpi). Oral, cloacal and skin swabs are probably poor samples to detect infection in clinically healthy frogs.

Keywords: Frog Virus 3, *Lithobates sylvaticus,* pathogenesis, *Rana sylvatica,* Ranavirus, wood frog

Introduction

The Iridoviridae is a family of large double-stranded DNA viruses that infect insects (*Iridovirus* and *Chloriridovirus* genera) and poikilothermic vertebrates (*Lymphocystivirus* and *Ranavirus* genera) (Chinchar *et al.*, 2002). Ranavirosis is a recognized emerging disease (Chinchar *et al.*, 2002) and, since 2008, one of only two amphibian pathogens reportable to the World Organisation for Animal Health (OIE, 2008). Frog Virus 3 (FV3), the type species of the genus *Ranavirus*, has been responsible for high mortality epizootics in fish, amphibians and reptiles (Chinchar *et al.*, 2009; Lesbarrères *et al.*, 2012). In North America (USA and Canada) at least 60 species of frogs, toads and salamanders (Anura and Caudata orders) are known to be susceptible to infection with FV3. Confirmed mortalities due to FV3 infection have been reported in 36 of those species (Miller *et al.*, 2011). Collapse in amphibian communities associated with the presence of the Common Midwife Toad virus, a species of *Ranavirus* present in continental Europe, may be the first indication that ranaviruses can result in population declines and, potentially, extirpations (local extinctions) (Price *et al.*, 2014). Mathematical population modeling provides further support for the ability of ranaviruses to cause local extinction: using susceptibility data and demographic parameters to predict the likelihood of local extinction of wood frogs (*Rana sylvatica* or *Lithobates sylvaticus*), Earl and Gray (2014) found that extirpation could occur as quickly as five years after introduction of *Ranavirus* sp to an isolated population.

Wood frogs, members of the large Ranidae family, are commonly used in the study of *Ranavirus* sp infection and were proposed as good experimental animal models by participants at the First International Symposium on Ranaviruses (Lesbarrères *et al.*, 2012). This species is widespread across North America, reaches the farthest north in Canada, and is sympatric with many other amphibians susceptible to *Ranavirus* sp infection. Adult wood frogs infected with a lethal dose of FV3 develop the gross and histologic lesions most consistently reported in other amphibians infected with various species of *Ranavirus* (e.g. Balseiro *et al.*, 2010 Bollinger *et al.*, 1999; Cullen & Owens, 2002; Jerrett *et al.*, 2015; Miller *et al.*, 2011): epidermal ulceration, dermal and visceral hemorrhages, and necrosis of the glomeruli, renal tubules and hematopoietic tissue in various organs (Forzán *et al.*, 2015).

Although the clinical signs, lethal dose and terminal lesions developed by adult wood frogs infected with FV3 have been described (Forzán *et al.*, 2015), the pathogenesis of the infection remains unknown. Research on aquatic African clawed frogs, *Xenopus laevis*, has partially elucidated the pathogenesis of *Ranavirus* sp infection in that species. A time-series experiment exposing adult *X. laevis* to a non-lethal dose of FV3 dissolved in water showed that viral DNA load (estimated by qPCR) was most abundant in intestinal tissue as soon as three hours post-exposure, suggesting that oral ingestion was the main route of infection (Robert *et al.*, 2011). The virus was also detected in blood, skin, liver and kidney. Evidence of active viral transcription (reverse transcription PCR) was most consistently detected in the intestine and kidney. The authors concluded that the intestine acts as a route of infection and transmission, and that the kidney is a main site of replication of FV3 in *X. laevis* (Robert *et al.*, 2011). Because lesions due to *Ranavirus* sp infection may vary depending on the pathogen and the host species (Hoverman *et al.*, 2011), further research must be conducted before extrapolating the findings in *X. laevis* to native North American species, such as the wood frog.

Our objective was to describe the pathogenesis of fatal FV3 infection in adult wood frogs by: 1) finding evidence of fecal-oral transmission, 2) describing the progression of lesions from time of infection to death, and 3) determining the organs or tissues that act as important foci of viral replication and shedding.

Materials and Methods

Origin and housing of experimental subjects

Wood frog adults were collected from an urban vernal pool (n=56) and a rural pond (n=3) in Prince Edward Island, Canada (May 17 and 22, 2012), and housed in accordance with guidelines of the Canadian Council for Animal Care (CCAC species-specific recommendations on: Amphibians and Reptiles, 2004 [http://www.ccac.ca/Documents/Standards/Guidelines/Add_PDFs/Wildlife_Amphibians_Reptiles.pdf). The frogs were housed individually or as small groups in polycarbonate tanks with shallow water dishes on bleach-free paper towel (Figure 1) and fed a combination of crickets (*Acheta domesticus*), mealworms (*Tenebrio molitor*) and earthworms (*Lumbricidae* sp). After 6.5 months of acclimation to captivity, the infection trial began (October 29). The temperature and humidity of the room where the animals were housed before and during the experiment were recorded daily, both as the value read immediately after entering the room in the morning, and as the minimum and maximum values of the previous 24 hours. The frogs were maintained at a fairly constant room temperature (average minimum-maximum of 20-22°C, range 18-23°C). Humidity varied substantially and reflected the seasonal ambient conditions (average minimum-maximum of 46-61% during acclimation and 28-44% during experimental trial). All mortalities that occurred during the acclimation months were examined grossly and histologically to determine cause of death, with a particular emphasis on any lesions suggestive of a ranavirus infection since there is no reliable method to detect subclinical infection in live animals. As none of the mortalities had any histological evidence of a ranaviral infection, we assumed that our wild-caught adults were free of the virus. After the 6.5 mo acclimation, 40 frogs were randomly assigned to one of seven infection groups (n=33 frogs) or served as uninfected controls (n=7 frogs) and moved to individual tanks.

Frog Virus 3 culture

The virus used in this study came from the original FV3 isolated in 1965 from a Northern leopard frog, *Rana* [*Lithobates*] *pipiens* (Granoff 1966). The isolate had been grown sequentially by various researchers in a variety of cells: Fathead minnow cells (FHM, at least 15-25 passages, G. Chinchar personal communication), Xenopus A6 cells and Baby Hamster Kidney fibroblasts (1-2 and 2 passages, respectively, J. Robert, personal communication). The virus stock used in this study was grown in Epithelioma papulosum cyprini cells (EPC, 2 passages) at room temperature (18-20°C). Titration of virus stock was performed in a six-well plate following standard methods to determine the number of plaque forming units (pfu) (Dulbeco 1953).

Infection, termination and sample collection

Three days after being moved to individual tanks, the frogs were randomly assigned to one of seven infection groups. The period from infection to euthanasia (end point) of each group was predetermined and ranged from 0.25 to 14 days post-infection (dpi). On infection day, each frog was inoculated orally with $10^{4.43}$ pfu of FV3 (Granoff 1966) contained in 0.25 ml of minimum essential media (MEM supplemented with 2% fetal bovine serum and 1% Antibiotic Antimycotic, Invitrogen) using a graded pipette (Figure 1). Uninfected control frogs (one per group) received the same volume of MEM. Frogs were checked two or three times daily to detect and record clinical signs; checks lasted from 1-2 h and were performed through the transparent wall of their enclosure except when the frogs were not readily visible (hiding under paper towel or cardboard tube) and it was necessary to open the lid and search for them. Crickets and mealworms were fed *ad libitum*; every other day prey consumed was recorded and any dead insects were removed; water was added as required and water dishes were changed every other day or sooner if they contained feces, skin sheds or dead insects. All handling started with the controls and continued through the infection groups; equipment (i.e. plastic gloves, metal forceps) was disinfected with sodium hypochlorite (5% bleach solution) after handling each frog or enclosure.

The frogs were euthanized at the predetermined end-point for their corresponding infection group: 0.25, 0.5, 1, 2, 4, 9 or 14 dpi. The exception was one frog that had to be euthanized 13 dpi because it showed clinical sings of serious illness compatible with terminal FV3 infection, i.e. petechial haemorrhages in the ventral skin with a focus of white necrosis (ulcer) in one of the legs (Figure 2), and severe depression with loss of normal posture (Forzán *et al.*, 2015). Euthanasia for all frogs consisted of immersion in a 10% solution of tricaine methanesulfonate (TMS, Syndel Laboratories LTD, Canada). Immediately after euthanasia the snout-vent (SV) length was measured and any abnormal gross findings recorded. Weight, being extremely variable due to hydration status, food in the stomach and urine in the gall bladder (Wright 2001), was intentionally not recorded. During post mortem examination, a cloacal swab and tissue samples of abdominal skin, left liver lobe and kidney were obtained, frozen at -80°C and later tested for ranavirus DNA by single-round PCR (Pisces Molecular, Boulder, Colorado, USA). The spleen of one frog in each infection group and of four control frogs was similarly obtained and preserved for PCR testing. The rest of the carcass and internal organs were preserved in 10% buffered formalin and, within two weeks of collection, trimmed and processed routinely for histological examination. Tissues examined were stained with hematoxylin-eosin and included one hand, one foot, a medial section of the head and jaw, a cross mid-shaft section of the thigh, and sections of the heart, lungs, abdominal fat body, liver (two sections), kidneys, urinary bladder, stomach, intestine, colon, spleen (except in those frogs which spleen was collected for PCR testing) and reproductive organs (ovary and oviduct or testicles). All procedures were approved by the Animal Care Committee of the University of Prince Edward Island (Protocol 12-014, file 6004702).

PCR for Ranavirus (FV3) DNA

Portions of each abdominal skin, liver, kidney and spleen sample were individually transferred into tissue lysis buffer, total DNA was extracted from all samples using a spin-column DNA purification procedure (Qiagen DNeasy 96, Qiagen, Valencia, California, USA), and tissues were tested for the ranavirus major capsid protein (MCP) gene with single round PCR amplification (Mao *et al.*, 1997), using the primers covering the same region of the MCP gene as the MCP1 assay recommended by the Aquatic Animal Health Code (5'–GACTTGGCCACTTATGAC-3’ and 5' –GTCTCTGGAGAAGAAGAA-3’) (OIE, 2012). For the fecal swabs, preparation was slightly different: lysis buffer was added into the sample tubes and vortexed at 55ºC four times within 1 hour, 10 g of carrier DNA was subsequently added to the lysis buffer and the lysis buffer was then transferred to newly labeled microfuge tubes for DNA extraction.

Immunohistochemical (IHC) staining

The tissues from one frog from each of the infection groups and one negative control frog were stained immunohistochemicaly to detect the presence of viral particles in affected tissues. Briefly, 5-µm sections were deparaffinised by immersion in two separate baths of xylene (2 min each), and three separate baths of 100% ethanol (1 m each), and rinsed in running tap water (1 min). After antigen epitope retrieval was achieved by boiling in Tris/EDTA pH 8.5 (TBS) solution for 20 min using an 850 W microwave, the slides were washed with tap water, and carefully dried, and a well was created around the tissue sections to hold the IHC solutions. Slides were washed three times with TBS, blocked with ELISA buffer containing casein (30 min at room temperature) and incubated (overnight at 4°C) with 100 µl of rabbit anti-Epizootic Hematopoietic Necrosis virus (anti-EHNV) antibody, diluted 1:500 in TBS (Reddacliff & Whittington, 1996), known to cross-react with FV3 (Ariel *et al.*, 2010). Slides were then washed three times with TBS, incubated in a solution of 0.3% hydrogen peroxide and 0.1% sodium azide in TBS (15 min) to inactivate endogenous peroxidises, washed three more times with TBS, and incubated (1.5 h at room temperature) with 100 µl of goat anti-rabbit-horseradish peroxidase conjugate antibody diluted in TBS. Following another three washes with TBS, the slides were developed with the addition of 100 µl of the chromogenic solution (0.005% 3-Amino-9-EthylCarbazole and 0.001% hydrogen peroxide in substrate buffer, 20 min at room temperature), then rinsed in running tap water, counterstained with hematoxylin (3 min), and rinsed again with tap water. Once dried, the slides were cover-slipped using aqueous mounting media. Non-specific binding and endogenous peroxidase control slides were produced by following the above method and omitting the primary and secondary antibodies, respectively. Staining for CD3 (monoclonal antibody clone CD3-12, Peter Moore, UC Davis, California, USA) and CD20 (polyclonal rabbit antisera Pierce Thermo Scientific, Rockford, Illinois, USA, PA5-16701) receptors on the surface of lymphocytes was attempted at Prairie Diagnostic Services (Saskatoon, Saskatchewan, Canada) in tissues from a single uninfected control frog.

Results

Frogs measured an average of 52.6 mm in SV length (median 52.9 mm, range 24.9-57.9 mm); all were male. With the exception of one frog that was euthanized one day ahead of its scheduled euthanasia (13 dpi) due to severe clinical signs consistent with terminal FV3 infection (Forzán *et al.*, 2015), all frogs survived until their respective euthanasia time. Gross and histological lesions and other findings present in frogs at the time of euthanasia are listed in Table 1. Gross lesions included petecchia in ventral skin, splenomegaly and hemorrhage in the wall of the gastrointestinal tract. Histologic lesions consistent with FV3 infection were more common in frogs euthanized towards the end of the trial (i.e. 9 and 14 dpi) than those euthanized soon after inoculation. Mild lesions in the skin (hemorrhages and mononuclear inflammation), oral mucosa (vascular necrosis), kidney (interstitial edema) and bone marrow (necrosis) were present in some frogs approximately 6-48 hours after oral inoculation with FV3 (0.25-2 dpi). Except for mild renal interstitial edema, lesions were mostly absent 4 dpi. Lesions were again observed 9 dpi, were the most severe 14 dpi and consisted of necrosis of hematopoietic and lymphoid tissues (bone marrow, spleen, thymus, kidney and liver), epidermal and glandular necrosis (skin), mucosal necrosis (oro-nasal cavity, tongue, lung and gastrointestinal tract), and glomerular and tubular necrosis (kidney) (Figure 3-5). Infiltrates of mostly mononuclear infiltrates in the oro-nasal mucosa and tongue were present in infected frogs at all sampling times, but their size increased with time and only after 9 dpi did they include necrotic/apoptotic cellular debris. Intracytoplasmic viral inclusions appeared first in the hematopoietic tissue (bone marrow and kidney, 9 dpi). At 14 dpi, viral inclusions were present in the oro-nasal mucosa, tongue epithelium, liver, pancreas, gastro-intestinal mucosa and, in very large numbers, epidermis.

Findings were classified as “incidental” (hepatocellular glycogen accumulation, peracute hepatic hemorrhage, old granulomas in coelomic cavity, and parasitic infections) if present in frogs from all infection groups and in uninfected controls. Periodic Acid Schiff (PAS) stain confirmed the accumulation of glycogen in hepatocellular cytoplasm. Of note was the paucity of extramedullary hematopoietic tissue in renal and hepatic parenchyma of those frogs that had no lesions and the uninfected controls.

Detection of *Ranavirus* sp DNA in tissues via PCR was, in general, consistent with the appearance of lesions. PCR for ranavirus DNA was negative in all samples (abdominal skin, liver, kidney, spleen and fecal swab) from frogs euthanized 0.25, 0.5, 1 and 2 dpi. The earliest positive PCR signal was recorded in the livers of two frogs at 4 dpi. At 9 and 14 dpi, all organs tested (i.e. skin, liver, kidney and spleen), as well as the fecal swab obtained immediately post mortem, were strongly positive for *Ranavirus* sp DNA (Table 2). Except for the livers of frogs euthanized at 4 dpi and the spleens of frogs euthanized at 9 and 14 dpi, all positive PCR signals corresponded to positive IHC staining (Table 3). All tissues from all control frogs (n=7) were negative for *Ranavirus* sp. DNA.

Immunohistochemical staining with anti-EHNV (*Ranavirus* sp) antibody was present in multiple tissues from frogs euthanized 9 and 14 dpi only (Table 3). Intracytoplasmic inclusion bodies found in the epidermis and bone marrow were strongly stained with IHC. Positive IHC staining was also present in the stomach contents of one frog euthanized 0.25 dpi and on the ciliated surface of the epithelium lining the duct connecting the Harderian gland with nasal cavity in one frog euthanized 0.5 dpi (a remnants of the oral inoculum). The spleens of all frogs consistently failed to stain with anti-EHNV IHC. No IHC staining for *Ranavirus* sp was present in any tissue from the uninfected controls.

Although a few scattered cells amongst mononuclear clusters in various tissues from the single uninfected control frog stained positive for CD3 (monoclonal CD3-12) and CD20 (polyclonal) antibodies, the vast majority of cells in those clusters, the splenic lymphoid follicles, and the thymus did not stain.

Discussion

Lesions caused by FV3 infection in these adult wood frogs were consistent with what we have recently described in fatally infected wood frogs (Forzán *et al.*, 2015). Although present in multiple organs, lesions were preferentially found in certain tissues: hematopoietic and lymphoid tissues, epidermal, renal tubular and mucosal epithelia, and vascular endothelia. Mild lesions consistent with damage to epithelium, endothelium and hematopoietic tissue occurred in the first 48 hours after inoculation and resulted in minimal petecchiae in skin of digits, mild renal interstitial edema and minute foci of bone marrow necrosis. These early lesions seemed to resolve and were no longer evident a few days after inoculation (4 dpi). When the lesions returned (9 dpi), they were more widespread and severe: Hemorrhages in the skin were larger, more frequent, found not only in digits, and accompanied by foci of epidermal necrosis. Hematopoieitic necrosis was more extensive in the bone marrow and present also in extramedullary hematopoietic tissue in the kidney and liver. Along with the exacerbation of lesions in the skin and hematopoietic tissue, necrosis of lymphoid tissue (spleen, thymus, oro-nasal and gastrointestinal mucosae), mucosal epithelium (oro-nasal cavity, tongue, stomach and intestine) and renal glomeruli and tubules developed. Our findings suggest that FV3 infection in adult wood frog first targets hematopoietic tissue in the bone marrow and endothelial cells in the skin causing very mild microscopic lesions. Then, after a few days during which lesions are no longer visible, FV3 causes severe lesions in medullary and extamedullary hematopoietic tissue, lymphoid tissue and epithelial cells of skin and mucosae throughout the body. Abundant IHC staining in the skin points to this tissue as a main site of viral replication and shedding. Replication in epidermal and mucosal epithelia and sheeding from skin and oral and gastrointestinal mucosae facilitate transmission through direct contact and a fecal-oral route. Because the experimental infection was conducted in the autum, outside of the seasons when mortalities from ranaviruses occur in the wild, i.e. spring and summer (Green 2002), the relevance of our findings could be questioned. Fortunately, the progression of disease we document here is consistent with the findings of a dose-dependent infection trial conducted in adult wood frogs in mid-spring (Forzán *et al.*, 2015).

Our conclusions are based on a combination of visible histopathologic changes and detection of viral presence through indirect methods, i.e. immunohistochemical staining and PCR detection of DNA. In the late stages of infection, each technique supported the findings of the other two: in most instances histologic evidence of necrosis in a tissue was consistent with positive IHC staining and a positive PCR signal. A notable exception to this agreement was the lack of IHC staining in the spleen of frogs that had obvious necrosis of the lymphoid tissue and a positive PCR signal. PCR is, of course, much more sensitive than IHC, so lack of staining could simply be the result of insufficient amount of viral antigen in the tissue to produce visible IHC staining. A more sensitive technique, such as in situ hybridization (Jessie *et al.*, 2004), might have been more successful. Alternatively, it could be that the virus was indeed absent from the spleen and the damage to its lymphoid tissue was indirectly due to FV3 infection. An induction of apoptosis through an increase of pro-apoptotic cytokines such as tumor necrosis alpha, which is upregulated in African clawed frogs, *Xenopus laevis*, infected with *Ranavirus* sp (Morales *et al.*, 2010), could have produced lymphoid necrosis without actual viral presence in the tissue. A similar effect at a distance could explain why PCR signals were not detected until 4 dpi, when the liver became positive. Before then, although mild histologic lesions were present in several tissues, IHC and PCR were negative. Because of the small size of most organs, only a few tissues were tested by PCR. Perhaps if we had tested bone marrow, or the skin from digits instead of abdomen, positive PCR signals would have been detected earlier in the infection.

A consistent finding in all frogs, infected or uninfected controls, was the glassy appearance of the cytoplasm in hepatocytes. This was interpreted as an abundance of glycogen (confirmed with PAS stain) and was thought to reflect the time of year when the experiment was conducted: mid to late autumn. The frogs, even with no access to outdoor light and maintained at a fairly constant temperature, seemed to have been able to sense the change in seasons and duly prepared for hibernation. Because wood frogs must tolerate freezing temperatures during winter, cryoprotectants, of which glucose is perhaps the most important, are crucial for survival (Costanzo *et al.*, 1993). Reaching the concentration of glucose necessary to prevent freezing damage to all tissues depends on previous accumulation of sufficient glycogen in the liver (Storey & Storey, 1986). Wild wood frogs in spring have much lower concentrations of liver glycogen (100-200 µmol/g fresh weight) than their counterparts in autumn (700-1000 µmol/g fresh weight) (Jenkins *et al.*, 2005). Glycogen accumulation, which renders livers friable, could also explain the presence of peracute (handling-associated) hemorrhages in several frogs, including one uninfected control. Neither the glycogen accumulation nor the liver hemorrhages were, therefore, considered to be associated with FV3 infection but incidental findings associated with the natural history of wood frogs. Other incidental findings, inevitable when working with wild-caught adults, included intestinal nematodiasis and skeletal muscle ichthyophoniasis. Nematode infection was mild, perhaps partly because of the deworming of all frogs five months before to treat an intense pulmonary rhabdiasis (0.02mg of Ivermectin per frog, transcutaneous). The small granulomas found on visceral serosal and adventitial surfaces of some frogs were likely associated with larval migration (in some, nematode larvae were still present). Ichthyophoniasis is not uncommon in wild-caught frogs from Prince Edward Island (Canadian Wildlife Health Cooperative necropsy records, unpublished) and has been reported also in wood frogs from Québec (Mikaelian *et al.*, 2000). Although massive infections of *Ichthyophonus* fungi have been documented as the cause of death of green frogs (*Rana* [*Lithobates*] *clamitans*) and bullfrogs (*R.*[*L.*] *catesbeiana*), mild endemic infections with little to no inflammatory reaction are more commonly observed (Green *et al.*, 2002). Nematodiasis, nematode larva-associated granulomas, and ichthyophonosis had no apparent deleterious effect on the health of the wood frogs involved in our experimental trial. Avoiding incidental infections with parasites or fungi in experimental subjects would require captive breeding, or at least captive-rearing from eggs. No captive-reared, let alone commercially available specific-pathogen-free, wood frogs are available at present, but captive rearing from eggs or tadpoles is possible

(Forzán *et al.*, 2015). To ensure that wild-caught frogs are not already infected with a specific pathogen in question, necropsy and histopathological examination of all mortalities in captivity, along with PCR or other specific ancillary tests, are indispensable. Based on such an examination of mortalities prior to the experimental trial, and from the PCR, histopathology and IHC staining of all controls, we are confident that our experimental subjects did not harbor a natural infection with *Ranavirus* sp. prior to the trial.

The pathogenesis of infection of adult wood frogs with FV3 resembles what occurs in other adult frogs infected with this or other species of *Ranavirus*. Adult Australian tree frogs (*Litoria splendida* and *L. caerulea*) that succumb to natural infections with Mahaffey Road (Bohle-like) virus developed widespread necrosis of epidermal and mucosal epithelia and lymphoid and hematopoietic tissues (Jerrett *et al.*, 2014). Unlike wood frogs in which necrotizing vasculitis was uncommon and which lacked any evidence of involvement of the central or peripheral nervous tissues, widespread vasculitis with endothelial necrosis, meningoencephalitis, myelitis and ganglioneuritis were prominent lesions reported in Australian tree frogs (Jerrett *et al.*, 2014). Based on IHC staining, Bohle-like virus infection in Australian tree frogs was strongly endotheliotropic, while FV3 in wood frogs preferentially targeted hematopoietic, lymphoid and epithelial tissues. In African clawed frogs, *Xenopus laevis*, infected with FV3 and euthanized at various times post-infection in a manner resembling the methods of our study, a strong PCR signal for viral DNA was detected in the skin 1 dpi and in the kidney 3 and 7 dpi, but it was absent or barely detectable in the liver at similar times (Robert *et al.*, 2011). Comparable findings were present in our wood frogs if we include histopathology and IHC staining results: mild lesions in the skin 1 dpi and strong DNA signals in the kidney 9 dpi. Unlike *X. laevis*, no DNA was detected in wood frogs 1 dpi, even though mild petecchiae were observed histopathologically. A possible explanation is preferential viral targeting of distal capillaries at early stages of infection: the lesions (petecchiae) 1 dpi were observed only in the skin of digits, while our sample for PCR testing was obtained from the ventral abdomen, where no lesions were present. Also unlike *X. laevis*, wood frog livers were positive for viral DNA as early as 4 dpi and, along with skin and kidney, strongly positive 9 dpi. Different tissue tropism could be due to characteristics of each host species. However, given the histopathological appearance and IHC staining of these tissues, the positive PCR signal in the liver and kidney in wood frogs was at least partly due to virus in local hematopoietic and lymphoid tissues and, in the case of the liver, resident melanomacrophages. Histopathology was not performed in the *X. laevis* study, so the precise location of the virus in the tissues was not determined. Perhaps, as lymphoid tissue is often present in the kidney of *X. laevis* (Wiechmann & Wiechmann, 2003), viral DNA detected in renal tissue corresponded to lymphocytes infected with FV3. Whether differences in tissue tropism suggested by comparing our study with those in Australian tree frogs or African clawed frogs are due to characteristics specific to the host, the pathogen or both would require experimental exposure of adults from each species of frog to the various *Ranavirus* spp.

One of the challenges in the study of disease ecology of *Ranavirus* spp is the lack of a sensitive diagnostic test that can detect infection in live animals. Cloacal and oral swabs, toe and tail clips have been used in an attempt to diagnose infection through non-lethal sampling (e.g. Gray *et al.*, 2012; Greer & Collins, 2007). Studies on the validity of PCR tests on samples that can be obtained from live animals have shown a failure to detect pre- or subclinical infections: tail clips in salamanders are negative except during active viremia (Greer & Collins, 2007); tail clips and oral and cloacal swabs of bullfrog tadpoles fail to identify approximately one fifth of infected individuals (Gray *et al.*, 2012). Our findings indicate that PCR on cloacal swabs of wood frogs fails to detect viral DNA until just a few days before death (when clinical signs become evident) and that survivors do not seem to shed detectable virus as early as two weeks after clinical signs disappear (Forzán *et al.*, 2015). Sampling of liver is appropriate in wood frogs, as it allows viral DNA detection earlier than other commonly sampled organs (skin, kidney and spleen). Further studies on non-lethal alternatives to detect *Ranavirus* sp infection are urgently needed. Until then, any non-lethal sampling of infected but clinically healthy individuals is likely to yield a false negative result.

We report a progression of lesions in adult wood frogs infected with a lethal dose of FV3. Mild lesions are first detected around 1 dpi in the skin (probably associated with damage to capillary endothelia) and bone marrow. Severe lesions arise approximately a week post-infection and consist of necrosis of medullary and extramedullary hematopoietic tissue, lymphoid tissue in spleen and throughout the body, and epithelium of skin and mucosae. The skin is an important site of viral replication and shedding. Oral and gastrointestinal mucosae and renal tubular epithelium are also important sites of viral replication. Direct contact (skin) and fecal-oral contamination are likely effective routes of transmission and, thus, skin and cloacal swabs may be reliable sources of ante-mortem diagnostic samples but only in late stages of disease, soon before or at the time of appearance of clinical signs. Oral, cloacal and skin swabs are inadequate samples to detect infection in clinically healthy frogs.

Acknowledgements

The authors thank Dr Alexandra Reid, who kindly provided the isolate of FV3 used in the infection, Dr Dale Godson and Ms Melissa Koehnlein for performing the lymphocyte immunostaining, Drs Marion Desmarchelier, Shannon Martinson and Jonathan Spears, Mr Chris MacQuaid and Mr Maciez Zawadzki, and in particular Dr Jessica Thompson and Ms Sara Vazquez Quiroga, whose efforts were indispensable to the successful rearing of the wood frogs. This work was partly funded by an Alexander Graham Bell Graduate Scholarship-Doctoral and the Canadian Wildlife Health Cooperative (formerly the Canadian Cooperative Wildlife Health Centre).

Table V - 1. Histologic lesions and other findings present in adult wood frogs, *Rana sylvatica*, orally infected with $10^{4.43}$ pfu of Frog Virus 3 (*Ranavirus* sp) and euthanized at different days post-infection (dpi, number of frogs with lesion present/total number of frogs in group; empty cell indicates that the lesion was absent in all frogs in group). Control frogs (Ctl, n=7) received a placebo; one control frog was euthanized at each of the end points (0.25, 0.5, 1, 2, 4, 9 and 14 dpi). Findings evident grossly as well as histologically are underlined.

Organ	**Lesion**	**dpi**							
		0.25	**0.5**	**1**	**2**	**4**	**9**	**14**	**Ctl**
Skin[a]	Hemorrhage	1/5	1/5	2/5	1/5		2/5	2/3	
	Epidermal necrosis						3/5	2/3	
	Epi/dermal inflammation		2/5	3/5	1/5	1/5	3/5	3/3	
	Intracytoplasmic inclusion bodies							2/3	
Bone Marrow	Necrosis		1/5	2/5			3/5	3/3	
	Intracytoplasmic inclusion bodies						1/5	2/3	
Oral/nasal cavity and tongue	Mucosal necrosis				1/5		3/5	3/3	
	Mononuclear infiltrates (mucosa/submucosa)	4/5	4/5	5/5	5/5	5/5	3/5	3/3	
	Intracytoplasmic inclusion bodies						1/5	3/3	
Salivary gland	Necrosis						0	1/3	
Thymus	Necrosis						1/3	3/3	
Blood vessels	Fibrinoid necrosis/vasculitis			1/5			2/5	3/3	
Liver	Hematopoietic tissue necrosis						5/5	3/3	
	Gall bladder necrosis							1/3	
	Intracytoplasmic inclusion bodies							1/3	
Pancreas	Necrosis		1/5				1/5	1/3	
	Intracytoplasmic inclusion bodies							1/3	
Adipose tissue	Hemorrhage or necrosis							1/3	
Kidney	Glomerular necrosis						2/5	2/3	
	Tubular degeneration/necrosis						3/5	1/3	
	Hematopoietic tissue necrosis						5/5	2/3	
	Interstitial edema			2/5		3/5			
	Proteinuria			1/5					
	Intracytoplasmic inclusion bodies						1/5		
Urinary bladder	Mucosal necrosis							1/3	
Spleen	Lymphoid/hematopoietic necrosis						5/5	3/3	

Lung	Alveolar/parabronchial epithelial necrosis						2/5	2/3	
Gastrointestinal tract	Mucosal necrosis						1/5	2/3	
	Submucosal hemorrhage							2/3	
	Intracytoplasmic inclusion bodies							2/3	
Incidental findings	Hepatocellular glycogen accumulation	5/5	5/5	5/5	5/5	5/5	5/5	3/3	
	Hepatic parenchymal (handling) hemorrhage	1/5	2/5	2/5	3/5	2/5	1/5		1/7
	Hepatocellular necrosis		1/5	1/5	1/5	1/5		1/3	
	Granulomas in serosa/adventitia	3/5	2/5	2/5	2/5	3/5	1/5		2/7
	Eosinophilic enteritis	3/5	1/5			1/5	3/5	1/3	3/7
	Intestinal nematodes	2/5	2/5		1/5	2/5		2/3	2/7
	Colonic protozoans	2/5	3/5	3/5	3/5	4/5	1/5	1/3	3/7
	Skeletal muscle *Ichthyophonus* sp.	3/5	3/5	5/5	1/5	2/5	1/5		4/7

[a] Lesions present mostly or solely in palmar/plantar surfaces of digits.

Table V - 2. Positive PCR results for presence of DNA of *Ranavirus* sp. in the tissues of adult wood frogs, *Rana sylvatica*, euthanized at different times (days post-infection, dpi, number of frogs with positive PCR results/total number of frogs in group) after oral infection with $10^{4.43}$ pfu of Frog Virus 3 (*Ranavirus* sp).

Organ or sample	**PCR (+)[a] (dpi)**						
	0.25	0.5	1	2	4	9	14[b]
Abdominal Skin	0/5	0/5	0/5	0/5	0/5	**5/5**	**3/3**
Liver	0/5	0/5	0/5	0/5	**2/5**	**5/5**	**3/3**
Kidney	0/5	0/5	0/5	0/5	0/5	**5/5**	**3/3**
Spleen	0/1	0/1	0/1	0/1	0/1	**1/1**	**1/1**
Fecal swab	0/5	0/5	0/5	0/5	0/5	**5/5**	**3/3**

[a] Positive results represent signals ranging from “positive” to “strongly” or “very strongly positive”. “Weakly positive” or “very weakly positive” signals were interpreted as negative.

[b] One frog euthanized at 13 dpi due to appearance of terminal clinical signs

Table V - 3. Immunohistochemical staining of tissues from selected adult wood frogs, *Rana sylvatica*, orally infected with $10^{4.43}$ pfu of Frog Virus 3 (*Ranavirus* sp) and euthanized at different days post-infection (dpi) with anti-Epizootic Hematopoietic Necrosis Virus (*Ranavirus* sp) (Reddacliff & Whittington, 1996). Tissues that had a positive PCR signal for *Ranavirus* sp DNA are indicated by an asterisk (*).

Organ	**dpi**						
	0.25	0.5	1	2	4	9	14
Skin	-	-	-	-	-	*+	*+
Bone Marrow	-	-	-	-	-	+	+
Oral/nasal cavity and tongue	-	-[a]	-	-	-	+	+
Salivary gland	-	-	-	-	-	-	+
Thymus	-	-	-	-	-	-	+
Blood vessels	-	-	-	-	-	+[b]	+[b]
Liver	-	-	-	-	*-	*+	*+
Pancreas	-	-	-	-	-	+	-[c]
Adipose tissue	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	*+	*+
Urinary bladder	-	-	-	-	-	-	-
Spleen	-	-	-	-	-	*-	*-
Lung	-	-	-	-	-	+	-[d]
Gastrointestinal tract	-[e]	-	-	-	-	+[f]	+[f]

[a] Cilia in the duct connecting nasal cavity with Harderian gland were positive (the inoculum fluid must have traveled up the duct after oral dosing).

[b] Positive staining of endothelial cells in liver at 9 and 14 dpi and in lung at 14 dpi.

[c] Possibly a false negative because of location of tissue on the slide (close to the edge of the well). It is possible that the antibodies or chromogen did not reach the tissue at the right concentration to produce positive staining.

[d] Abundant background staining precluded a conclusive interpretation.

[e] Positive staining of the gastric contents, likely corresponding to the oral inoculum administered only 6 hours before.

[f] Positive staining in stomach but no conclusive staining in small or large intestine (possibly a false negative because of location of tissue on the slides, towards the edge of the well).

Figure V - 1. Individual housing of adult wood frogs, *Rana sylvatica* (upper left and right), oral inoculation with $10^{4.43}$ pfu of Frog Virus 3 (lower left) and euthanasia in tricaine methanesulfonate (lower left).

Figure V - 2. Gross lesions observed in adult wood frogs, *Rana sylvatica*, orally inoculated with $10^{4.43}$ pfu of Frog Virus 3 and euthanized 14 days later. Hemorrhage in the skin of the inguinal region, ventral thigh regions and both feet; an ulcer (white arrow) is present in the hemorrhagic skin of the right thigh (top). Dark red (hemorrhagic) intestinal wall (white arrow) and petecchia in pale testicles (black arrow, lower left). Splenomegaly (black arrow) and hemorrhage (white arrow) in the wall of the stomach (lower right).

Figure V - 3. Spleens from adult wood frogs, *Rana sylvatica*, orally inoculated with $10^{4.43}$ pfu of Frog Virus 3 and euthanized 4, 9 and 14 days later. Well-defined lymphoid nodules (black arrow) and melanomacrophages (dark brown cells, black circle) are evident in the spleen of the control frog (left). Infected spleens show chronologically progressive shrinkage and necrosis of the lymphoid nodules, and reduction in the number of melanomacropahges. Hematoxylin and eosin stain.

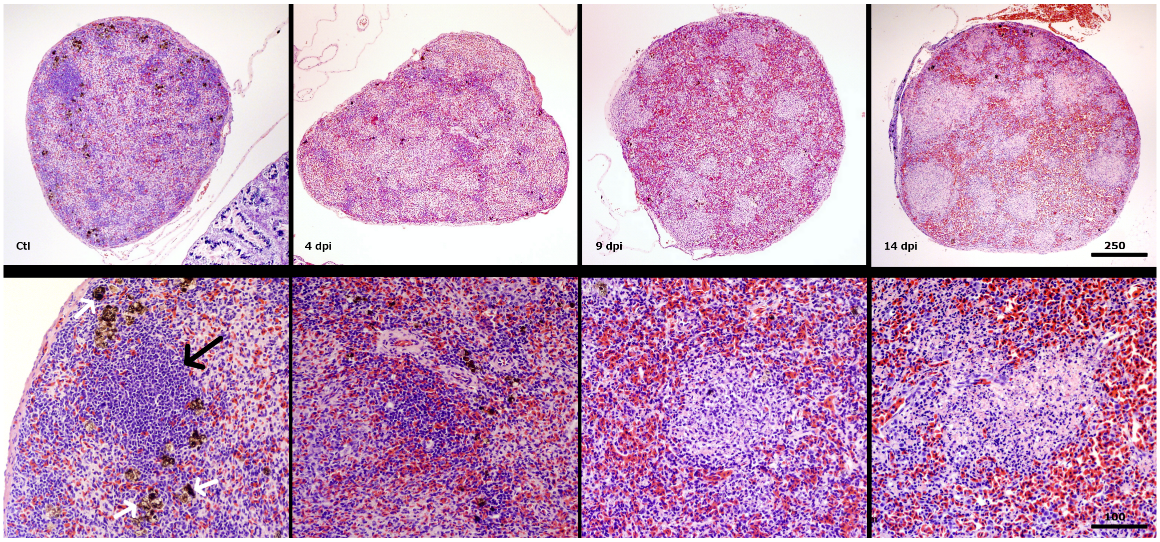


Figure V - 4. Nasal mucosa of wood frogs, *Rana sylvatica*, orally inoculated with $10^{4.43}$ pfu of Frog Virus 3 and euthanized 14 days later. Submucosal lymphocyte nodule, more prominent in frogs as infection progressed, includes a small area of necrosis (black arrow; left top and bottom). Hemorrhage in submucosal lymphocyte nodule (black arrow; top middle) and intracytoplasmic inclusion bodies that stain with anti-ranavirus antibodies (anti-Epizootic Hematopoietic Necrosis virus, *Ranavirus* sp) in the overlying epithelium (bottom middle and right). An uninfected control is shown on the top right for comparison. Hematoxylin and eosin stain (top row and bottom left and middle); immunohistochemical stain (bottom right).

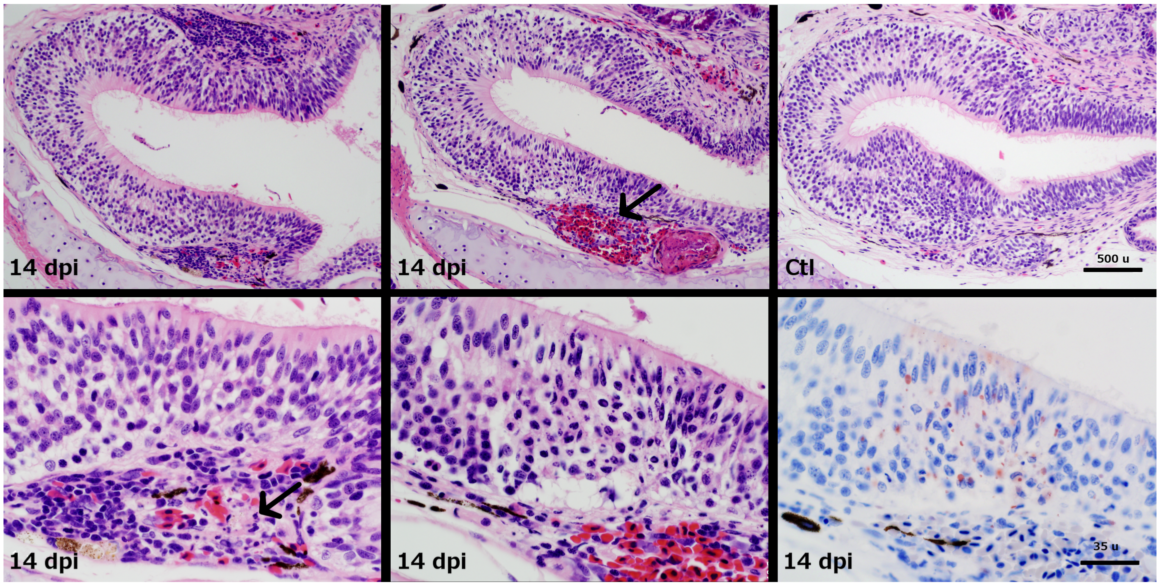
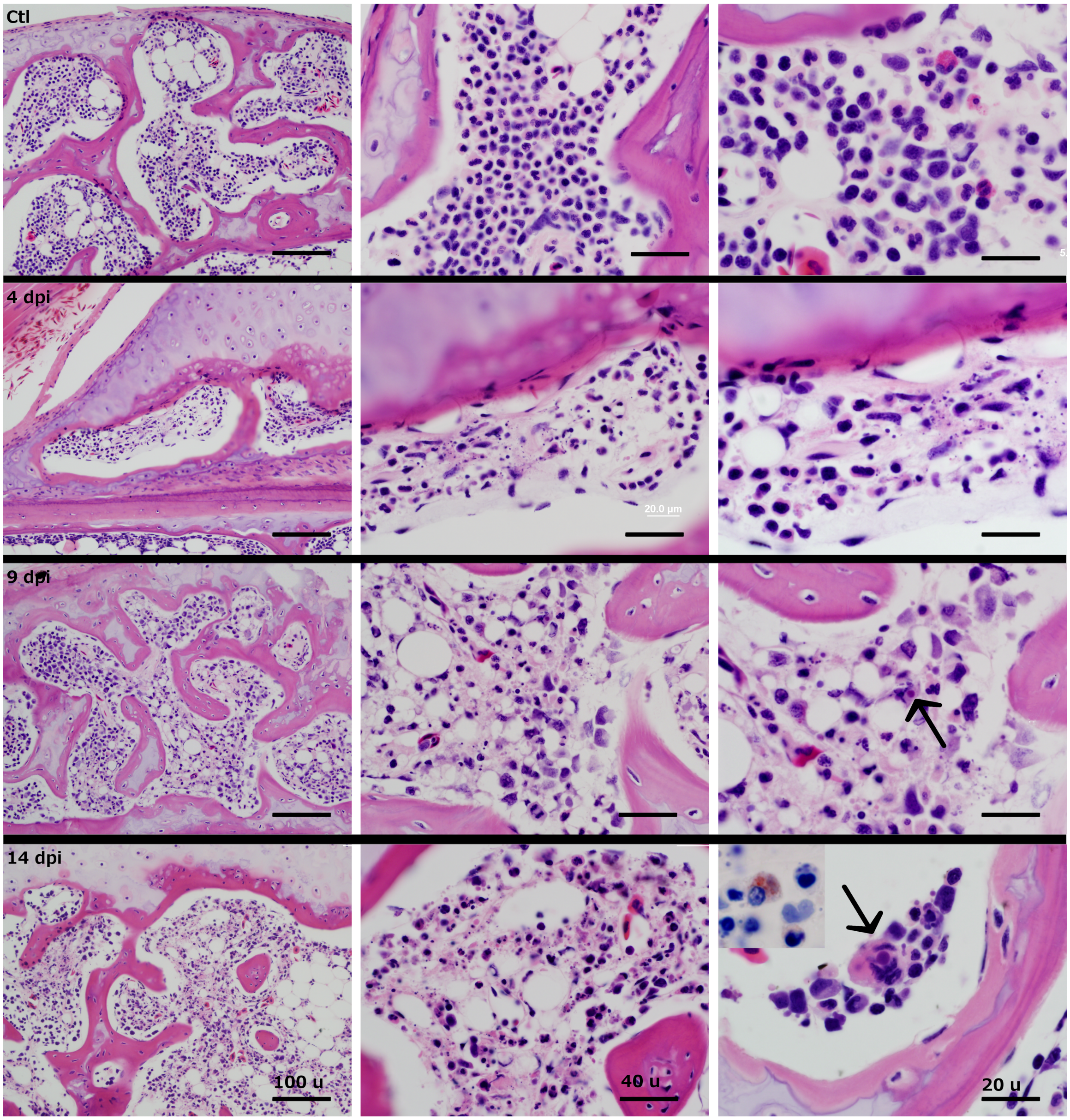


Figure V - 5. Bone marrow of wood frogs, *Rana sylvatica*, orally inoculated with $10^{4.43}$ pfu of Frog Virus 3 and euthanized 4, 9 and 14 days later (dpi), and an uninfected control (Ctl). Necrosis of the hematopoietic tissue increases in severity with time. Intracytoplasmic inclusion bodies, probably of viral origin, are present in a multinucleated cell (either an osteoclast or a megakaryocyte; black arrow, bottom right) and an unidentified cell (black arrow, second to bottom row, right). Anti-ranavirus antibodies (anti-Epizootic Hematopoietic Necrosis virus, *Ranavirus* sp) depict intracytoplasmic inclusions in a hematic cell (insert, bottom right). Hematoxylin and eosin stain (all but insert); immunohistochemical stain (insert bottom right).



CONCLUSION

It's not that easy bein' green
Having to spend each day the color of the leaves
When I think it could be nicer being red or yellow or gold
Or something much more colorful like that.
It's not easy bein' green
It seems you blend in with so many other ordinary things
And people tend to pass you over 'cause you're
Not standing out like flashy sparkles in the water
Or stars in the sky...
Kermit The Frog (Joe Raposo, composer, 1970)

Amphibians, the most threatened vertebrate Class worldwide, face severe problems. Habitat loss is the most significant and, possibly, the most difficult problem to mitigate given our hunger to forever expand our own habitat. Infectious diseases, which spread has been at least partially facilitated by human activities, are other significant threats. In 2008, the World Organization for Animal Health (OIE) for the first time added amphibian pathogens to the list of notifiable diseases. Chytridiomycosis, caused by *Batrachochytrium dendrobatidis* and, recently, *B. salamandrivorans*, have devastated frog and toad populations in Central American and Australia, and are decimating the fire salamander population in The Netherlands. In North America and Europe, ranaviruses cause large mortalities of amphibians, reptiles and fish. Interdisciplinary research teams are currently working on elucidating the pathogenesis and ecology of these significant pathogens, as the first step in developing mitigating or preventive measures.

The present work provides information on the maintenance of wood frogs (*Rana sylvatica*) in captivity, describes the dose-dependent clinical signs and lesions of adult wood frog orally inoculated with Frog Virus 3 (FV3, *Ranavirus* sp, Iridoviridae), reviews the literature on clinical pathology of amphibians, provides hematological reference intervals for adult wood frogs, describes the changes in the hematological profile of wood frogs infected with FV3, and the progression of FV3-associated histological lesions through time.

The captive housing and rearing of wood frogs proved labor intensive and expensive but in general terms was quite successful. The description of methods and procedures employed, as well as the failures and problems experienced during the year the frogs were housed at the Atlantic Veterinary College should be useful to researchers who need to house the species in their own laboratories. Successfully maintaining wood frogs at all stages of development will be particularly useful if the species is to become one of the main experimental subjects in the study of amphibian diseases.

The studies reported here are the first to perform dose-dependent and time-course trial infections with FV3 on adult wood frogs. Both trials are basic in the development and characterization of this ranavirus-wood frog model, and important steps in the research of ranavirus infection in North American frogs. Oral inoculation, developed for these studies, was easily performed and allowed for the administration of precise doses. Study results include clinical signs, median survival time, probability of death at a given dose, viral shedding in feces and skin sheds, gross and histological lesions and immunohistochemical staining results under controlled laboratory conditions. These findings provide transmission, infection and mortality estimates that could be incorporate into ranavirus disease models, and facilitate the design of experiments to investigate the pathogenesis of ranavirus infection in North American frogs.

The hematological reference intervals provided here can be used to evaluate the health of captive wood frogs and, with caution, the health of free-ranging individuals. Along with the reference intervals, the present work describes alternations in the hematological profile associated with a fatal infection with FV3. Adult wood frogs seem potentially capable of mounting an immune response to FV3 infection involving cells of the adaptive immune system - namely circulating lymphocytes. Further studies will determine whether an immune response can indeed be induced and whether prophylactic immunization would be effective in protecting amphibians against subsequent exposure to this emerging pathogen.

A fatal infection with FV3 in adult wood frogs is initially evidenced by mild lesions in the skin and bone marrow, which occur around 24 hours after infection. Lesions in the skin are likely associated with damage to capillaries. Approximately a week post-infection severe lesions consisting of necrosis of medullary and extramedullary hematopoietic tissue, lymphoid tissue in spleen and throughout the body, and epithelium of skin and mucosae, develop. The skin is an important site of viral replication and shedding. Oral and gastrointestinal mucosae, and renal tubular epithelium are also important sites of viral replication. Direct contact (skin) and fecal-oral contamination are likely effective routes of transmission and, thus, skin and cloacal swabs could be reliable sources of ante-mortem diagnostic samples but only in late stages of disease, soon before or at the time of appearance of clinical signs. Oral, cloacal and skin swabs are inadequate samples to detect infection in clinically healthy frogs.

Further questions and avenues of research that arise from these findings include the evaluation of immune response to FV3, the exploration of protective inoculations (vaccinations), and the further characterization of pathogenesis of a fatal infection, perhaps using more sensitive detection techniques (such as *in situ* hybridization). Although infectious diseases may not be the most imperative threats to amphibians everywhere, research that may contribute to their prevention or mitigation would certainly help amphibian populations worldwide, whether they are red, yellow, gold or the color of leaves.

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APPENDICES

CHAPTER TWO

Appendix A. Calculation of median Tissue Culture Infective Dose ($TCID_{50}$)

Based on instructions found in Virología Veterinaria, by Mohanty and Dutta, 1983. P.43

1. *Calculate proportional distance (PD)*

$$PD = \frac{\text{(dilution >50\% cytotoxicity)} - \text{(50\%)}}{\text{(dilution >50\% cytotoxicity)} - \text{(dilution <50\% cytotoxicity)}}$$

2. *Add PD to coefficient of dilution >50% cytotoxicity*
3. *Switch to absolute value and adjust for 1ml volume(instead of the original 0.1 ml used in the assay)*
4. *Result reported as $10^{x}TCID_{50}/ml$*

CALCULATION OF FV3 ISOLATE $TCID_{50}/ml$ (c=cytotoxicity)

Control (-)	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	**10^{-5}**	**10^{-6}**	10^{-7}	10^{-8}	10^{-9}	10^{-10}
-	*c*	*c*	*c*	*c*	*c*	**-**	**-**	-	-	-	-
-	*c*	*c*	*c*	*c*	*c*	***c***	**-**	-	-	-	-
-	*c*	*c*	*c*	*c*	*c*	***c***	**-**	-	-	-	-
-	*c*	*c*	*c*	*c*	*c*	***c***	**-**	-	-	-	-
-	*c*	*c*	*c*	*c*	*c*	***c***	**-**	-	-	-	-
-	*c*	*c*	*c*	*c*	*c*	***c***	**-**	-	-	-	-
-	*c*	*c*	*c*	*c*	*c*	***c***	**-**	-	-	-	-
-	*c*	*c*	*c*	*c*	*c*	**-**	**-**	-	-	-	-
**0%*	*100%*	*100%*	*100%*	*100%*	*100%*	***75%***	***0%***	*0%*	*0%*	*0%*	*0%*
Observations at end of trial, day6 post-infection (Oct 18, 2012) **Percentage of cytotoxicity per dilution*											

1. $PD = \frac{75 - 50}{75 - 0} = 25/75 = 0.3333$
2. $10^{-5} + 10^{-0.3333} = 10^{-5.33}$
3. $10^{5.33}/0.1\ ml = 10^{6.33}/ml$
4. **$10^{6.33}TCID_{50}/ml$**

Appendix B. Calculation of plaque-forming units (pfu)

1. Plaquing assay, as prepared by Kathleen Jones

Performed plates in duplicate, one using 37°C agar and one using agar cooled to 24°C to ensure temperature shock to cells can be avoided.

1. *One day before the assay, cells (EPC 83-2 P19) were seeded into 6-well plates at a regular ratio of 1:4. Monolayer was approximately 95% upon infection.*
2. *Serial dilute virus as follows:*
    - *a. A 1:100 dilution was prepared by adding 30µl viral stock to 2970µl MEM plain media.*
    - *b. Starting with the 1:100 dilution, prepared serial 1:10 dilutions by transferring 300µl of diluted virus to 2700µl MEM plain up to* 10^{-6} *.*
3. *Washed plates once with D-PBS (aka PBS-A in notebook).*
4. *Infected cells with 0.5 ml diluents of virus, from most dilute to most concentrate. The plates were gently rocked to spread the virus evenly over the monolayer.*
5. *Incubated the cells for 75 min RT to allow the virus to adsorb to the cells.*
6. *During incubation, prepared agarose overlay medium as follows.*
    - *a. Melted 2.5g of agarose in 50ml D-PBS using microwave. Allowed to boil then stirred multiple times until liquid was clear and quite viscous but full of bubbles. Cooled to 37°C in incubator.*
    - *b. Warmed 90 ml growth medium to 37°C in incubator.*
    - *c. Added10 ml 5% agarose to the 90 ml growth medium. Mixed very well.*
7. *Removed the virus-containing medium from the cells to maintain agar: media ratio.*
8. *Gently added 3 ml of 37°C agarose solution to each well, taking care not to dislodge any cells. Allowed agarose solution to cool to approximately 24°C and repeated with duplicate plates.*
9. *When the agarose was set (approx.10 min at RT), sealed plates with tape and parafilm and incubated at RT.*
10. *Checked plates daily until plaques have formed and not progressed.*

2. Counting the plaques, as done under supervision by Fred Kibenge

Based on instructions on Plaque Assay by Capital Biosciences (http://www.capitalbiosciences.com/product/print/plaque-assay-cd0 - the rest is not in the print out, but it is pasted to my lab book).

1. *Prepare a 0.03% solution of neutral red in PBS (1 ml 0.33% [w/v] neutral red stock + 10 ml PBS).*
2. *Add 1ml of the 0.03% neutral red solution to each of the wells and incubate for 2-3 hours [applied on day 4 post-infection and left to incubate for 24 hours in this assay. MF].*
3. *Remove the stain by aspiration (if there is any that hasn't been taken up by the gel [there wasn't, it all got absorbed]), and then invert the dishes to allow the plaques to clear.*
    a. *Neutral red is a vital stain and is therefore taken up by healthy cells but not by dead cells. Therefore, plaques appear as clear circles against a red or pink background [read on day 5 post-infection, that is day 6 post-seeding, MF].*
4. *Count the number of well-isolated plaques (choose a well with just under 100 plaques to be representative but avoid double-counting).*
5. *Use the following formula to determine the titer (pfu/ml) of your viral stock:*

pfu/ml = #plaques / (D x V)

when D=dilution factor and V=volume of diluted virus/well

CALCULATION OF FV3 ISOLATE pfu/ml in our assay

#plaques in best 2 wells (one incubated at 37°C and the other at 24°C, both read on day): 106 and 110 (counted by MF and KJ separatedly). **Average count: 108 plaques**

D=10^{-5} V=0.2ml

pfu/ml = 108 / (10^{-5} x 0.2) = 108 / (0.00001x0.2) = 108/0.000002

= 54,000 000 = 5.4x10^{7} OR $^{=}10^{7.73}$

FV3 isolate has $10^{7.73}$ pfu/ml (53,703,180 virus particles/ml)

Appendix C. Calculation of LD50: our wood frogs

Based on instructions found in Virología Veterinaria, by Mohanty and Dutta, 1983. P.43, and advice from Fred Kibenge

1. *Calculate proportional distance (PD)*

$$PD = \frac{(\text{pfu/frog} > 50\% \text{ mortality}) - (50\%)}{(\text{pfu/frog} > 50\% \text{ mortality}) - (\text{pfu/frog} < 50\% \text{ mortality})}$$

2. *Add PD to coefficient of dilution <50% mortality*
3. *Result reported as* $LD_{50} = 10^{x}$ *pfu/frog*

OR

2. LD_{50} *= PD X [(pfu>50% mort)- (pfu<50% mort)]+ (pfu<50% mort)*
3. *Result reported as* $LD_{50} = 10^{x}$ *pfu/frog*

CALCULATION OF FV3 ISOLATE LD_{50}***/frog in a 0.05 ml volume (m=mort)***

Control(-)	$10^{5.4}$	$10^{4.4}$	$10^{3.4}$	$10^{2.4}$	$10^{1.4}$	$\mathbf{10^{0.4}}$
-	*m*	*m*	*m*	*m*	-	-
-	*m*	*m*	*m*	-	-	-
-	*m*	*m*	*m*	-	-	-
-	*m*	*m*	*m*	-	-	-
-	*m*	*m*	-	-	-	-
**0%*	*100%*	*100%*	***80%***	***20%***	*0%*	*0%*
Morts by the end of trial, day 22 post-infection (Apr 30, 2013) **Percentage of morts per #pfu/ml*						

1. $PD = \frac{80 - 50}{80 - 20} = 30/60 = 0.5$
2. $10^{2.4} + {}^{0.5} = 10^{2.9}$
3. **$LD_{50} = 10^{2.9}$ pfu/frog**

OR

2. LD_{50} *= PD X [(pfu>50% mort)- (pfu<50% mort)]+ (pfu<50% mort)*

   LD_{50} *= 0.5 X [3.4-2.4]+2.4 = (0.5 x 1)+2.4 = 0.5+2.4 =* ***2.9***
3. ***$LD_{50} = 10^{2.9}$ pfu/frog (794.33 ≈ 800 virus particles/frog) using virus suspension of $10^{4.2}$ pfu/ml***

Appendix D. Calculation of LD50: Brunner et al.'s salamander tadpoles exposed to ATV ranavirus

Based on instructions found in Virología Veterinaria, by Mohanty and Dutta, 1983. P.43, and advice from Fred Kibenge

4. *Calculate proportional distance (PD) of pfu/ml of water (200ml) in which the tadpoles swam for 24 hrs (Brunner et al. 2005)*

$$PD = \frac{(\text{pfu/ml} > 50\%\ \text{mortality}) - (50\%)}{(\text{pfu/ml} > 50\%\ \text{mortality}) - (\text{pfu/ml} < 50\%\ \text{mortality})}$$

5. *Add PD to coefficient of dilution <50% mortality*
6. *Result reported as* $LD_{50} = 10^{x}$ *pfu/frog*

OR

4. $LD_{50} = PD \times [(\text{pfu} > 50\%\ \text{mort}) - (\text{pfu} < 50\%\ \text{mort})] + (\text{pfu} < 50\%\ \text{mort})$
5. *Result reported as* $LD_{50} = 10^{x}$ *pfu/frog*

CALCULATION OF FV3 ISOLATE LD_{50}***/tadpole (200 ml of water, concentrations of pfu/ml)***

Control(-)	10^{5}	10^{4}	$10^{3.5}$	10^{3}	$10^{2.5}$	$\mathbf{10^{2}}$
0/23	*51/56*	*51/56*	*47/56*	*25/54*	*16/56*	*2/57*
**0%*	*91.1%*	***91.1%***	*84%*	***46.3%***	*28.6%*	*3.5%*

Tiger salamander, Ambystoma tigrinum, tadpoles exposed to ATV ranavirus
**Percentage of dead/exposed*

OR

4. $LD_{50} = PD \times [(\text{pfu} > 50\%\ \text{mort}) - (\text{pfu} < 50\%\ \text{mort})] + (\text{pfu} < 50\%\ \text{mort})$
   $LD_{50} = 0.9 \times [3 - 3.5] + 3.5 = (0.9 \times -0.5) + 3.5 = -0.45 + 3.5 = 3.05$
5. $\mathbf{LD_{50} = 10^{3.05}}$ ***pfu/ml (1,122 ≈ 1000 virus particles/ml or 200,000/200ml)***